nature OUTLOOK

HUMAN PAPILLOMAVIRUS

30 August 2012 / Vol 488 / Issue No. 7413

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It is tempting to characterize the human papillomavirus story as a triumph of science. Over the course of nearly 40 years, a lethal cancer was found to be caused by a particular virus, and vaccines were developed and rolled-out across the world. If only other cancers proved as tractable.

But, as we chronicle in this Outlook, the tale of this virus is still being written. For one thing, these vaccines have not yet met their most stringent endpoint: prevent cervical cancer (page S4). Moreover, HPV vaccines are expensive and, because they require refrigeration and come in three doses, impractical for much of the developing world — which bears the biggest burden of cervical cancer (S2). More durable vaccines in development might prove more useful (S7).

Research is upending the basis of most cancer screening programmes: the Pap test. New methods to detect the virus directly could warn of impending disease earlier (S8).

Most young women become infected with the virus, yet many clear the virus naturally and never go on to develop cervical cancer. Researchers are investigating why this is so (S14). And HPV may not be alone among cancer-causing viruses. Harald zur Hausen — the scientist who received a Nobel prize for his work linking HPV to cancer — points to a couple of other culprits (S16).

Because of its close association with cervical cancer, HPV is generally considered to be a women's health issue. That's a mistake, argues Margaret Stanley: men and women both suffer from HPV-related illnesses so both sexes should be vaccinated (S10). The prevention of HPV infection, and ultimately cervical cancer, is very much a work in progress and it will be some time before we can consign HPV-related cancers to the history books.

We acknowledge the financial support of VACTIA – The Center for Vaccine Research and Immunology, The Finnish Medical Association and PreHdict in producing this Outlook. As always, *Nature* has full responsibility for all editorial content.

Herb Brody
Supplements Editor

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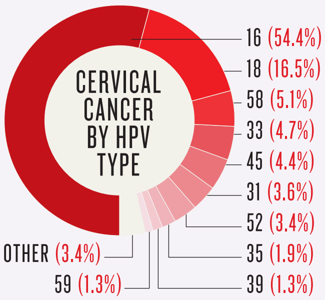
HPV: THE GLOBAL BURDEN

Human papillomavirus (HPV) has become synonymous with cervical cancer, but its actual footprint is much bigger, by **James Mitchell Crow**.

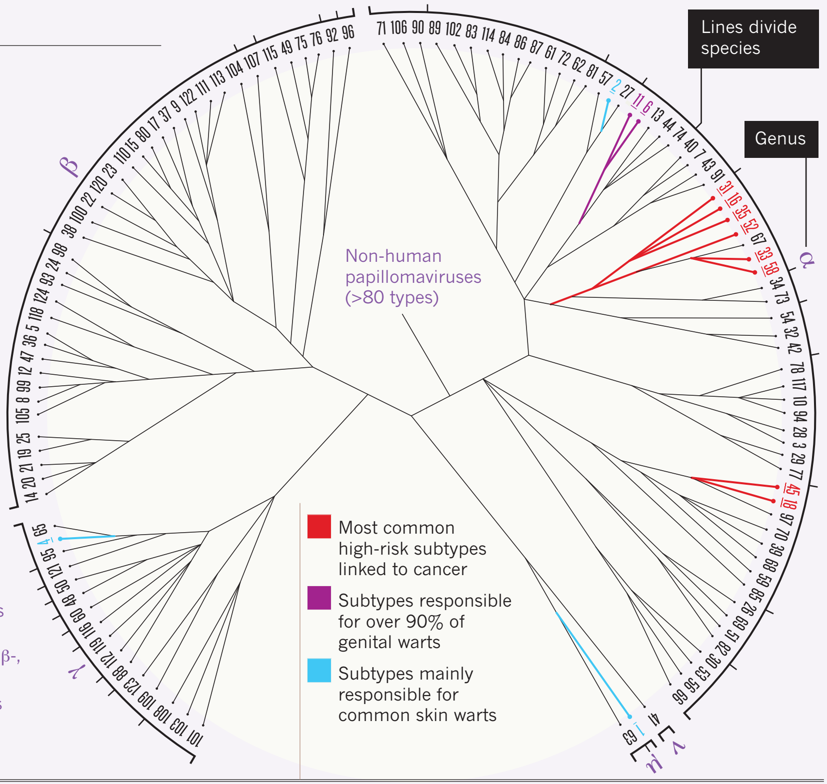
HPV COMES IN MANY FORMS

Tens of different papillomavirus types infect humans, but only a handful are harmful. Mapping HPV types by genus (right) reveals that certain species often cause simlar warts and lesions, with most of the HPV types that cause cancer coming from the same species. However, shared pathology doesn't always indicate close family ties; HPV types 1, 2 and 4, which all cause cause common skin warts, are distant relations.



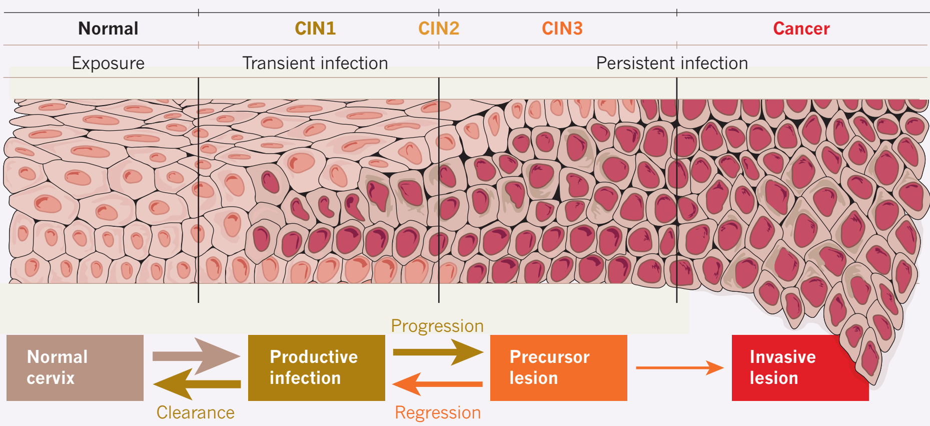
α, β, γ, μ, ν

HPV genera: α-papillomaviruses infect mucosal surfaces and skin, β-, γ-, μ- and ν-papillomaviruses only skin.

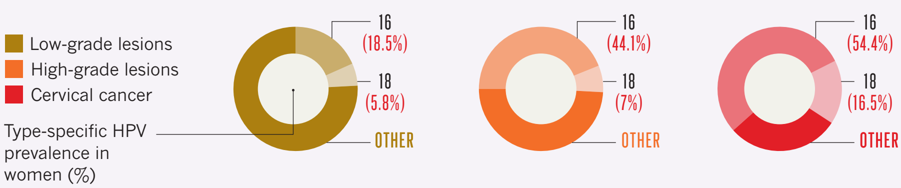


HOW HPV CAN LEAD TO CANCER

Although HPV infections are common, 90% of cervical infections are cleared within 2 years. If infection persists, abnormal cells can begin to appear. Only if these cervical cells cross the basal membrane and spread into the tissues beneth does the condition become cervical cancer.



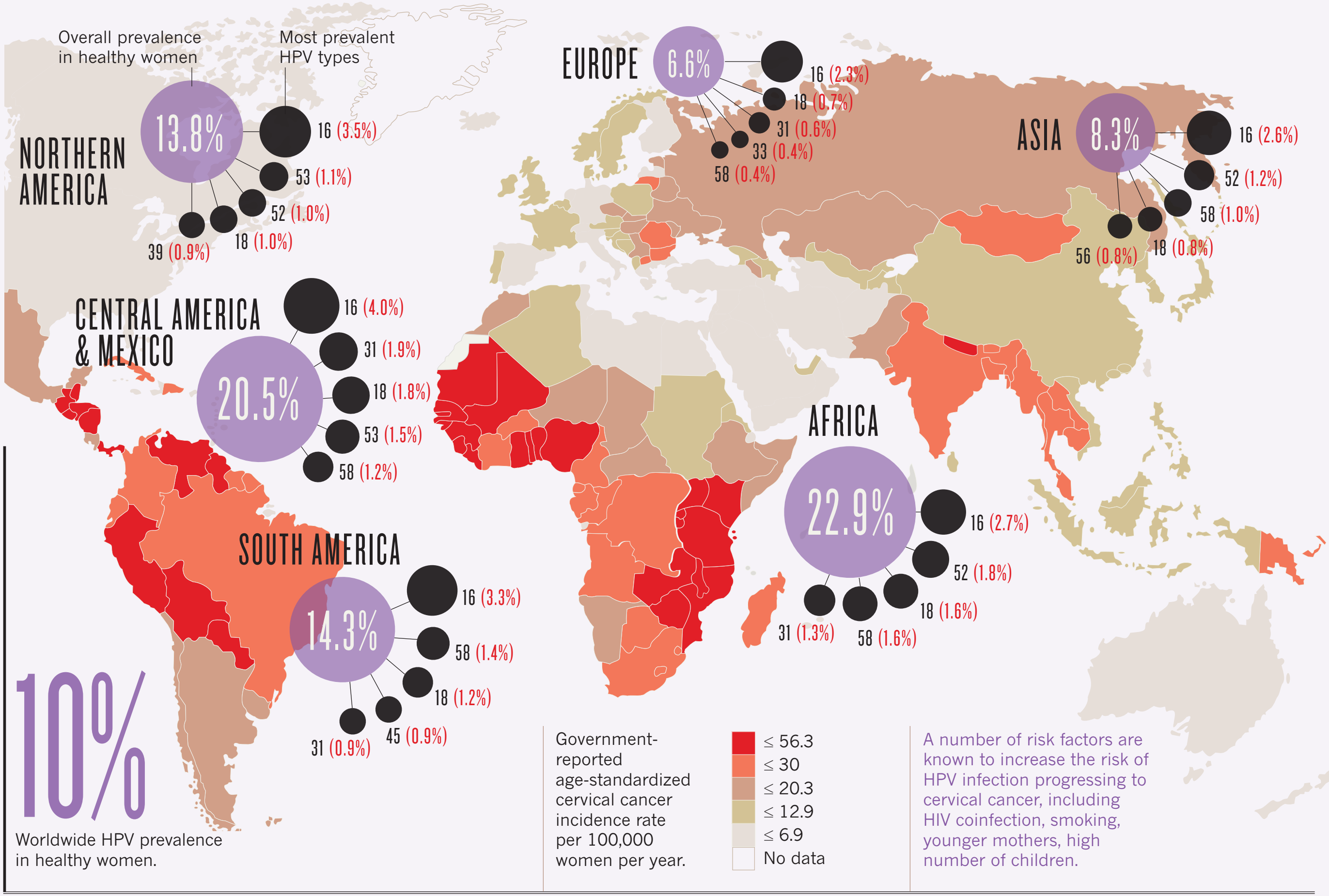
A few abnormally sized and oddly shaped cells on the surface of the cervix is classified as Cervical Intraepithelial Neoplasia 1 (CIN1), a low grade lesion that typically disappears within a few months without treatment. A large number of precancerous cells on the surface of the cervix that are distinctly different from normal cells is classified as CIN3. High-grade CIN3 lesions are still reversible spontaneously or through treatment.



Infection with certain HPV subtypes is more likely to lead to cancer than others. HPV types 16 and 18 make up only 2.7% and 1.1% of initial cervical infections respectively, but together account for 70% of cervical cancer cases.

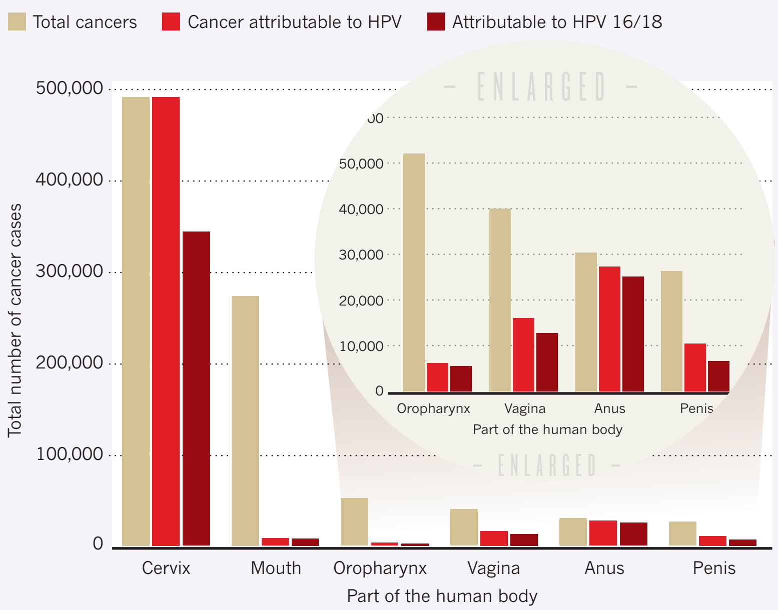
INFECTION RATES AND CANCER CASES

Cervical HPV infection rates vary around the world, as does the number of infected women who go on to develop cervical cancer.



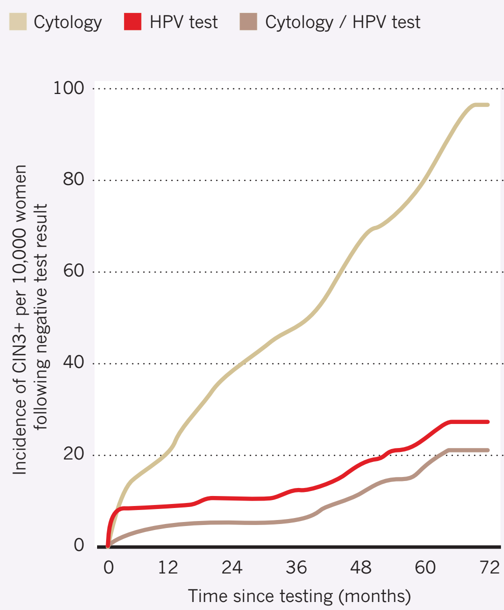
HPV AND CANCER

Although the overwhelming majority of cancers caused by HPV infection are of the cervix, infection with the virus can also lead to cancers in other parts of the body.



THE FUTURE OF HPV SCREENING

HPV tests are proving more effective than traditional cytology screens at catching early-stage infections that progress to high-risk lesions.



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VOISIN/PHANIE/REX FEATURES

CLINICAL APPROVAL

Trials of an anticancer jab

Two vaccines seem to be so effective in preventing HPV infection that mass vaccination has been introduced for girls. But will long-term studies show falls in cervical cancer?

BY JULIE CLAYTON

For Matti Lehtinen, a virologist and professor of public health at the University of Tampere, Finland, it was a setback to hear that the cause of cervical cancer was the human papillomavirus (HPV). He had spent several years on the wrong track — as had many scientists during the 1970s — investigating a different virus altogether: the herpes simplex virus (HSV). "It was very disappointing," he admits.

But Lehtinen turned adversity to advantage, and embarked on a career that is now seeing the science of HPV through to its ultimate application: cancer prevention. He was lead author on a paper that showed that infection with HPV type 16 is a risk factor for cervical cancer, and for the past ten years he has been lead scientific investigator on the Finnish arms of clinical trials for the two vaccines against HPV.

Both vaccines have now been approved, with the stated aim of preventing cervical cancer, which kills about a quarter-of-a-million women each year. Although successful, the vaccine trials did not demonstrate anticancer activity; approval was based on the assumption that eradicating HPV infection would reduce or eliminate the risk of cervical cancer. Lehtinen admits that proving this final step will require a long-term human study.

EARLY RESULTS

The first vaccine to enter phase III efficacy trials was Gardasil, produced by pharmaceutical giant Merck, based in Whitehouse Station, New Jersey. Gardasil contains peptides from four HPV types: 16 and 18, which account for at least 70% of cervical cancer cases globally; and 6 and 11, which are commonly associated with anogenital warts (see 'The global burden', page S2). The two Gardasil trials (Future I and II) enrolled 17,600 women across the Americas, Europe and the Asia–Pacific region, who received jabs between December 2001 and May 2003.

The second vaccine is Cervarix from GlaxoSmithKline, based in London, which began its phase III trial (Patricia) in May 2003. In total, 18,600 women were enrolled in 14 countries across the Asia–Pacific region, Europe, Latin America and North America.

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Cervarix contains peptides from HPV types 16 and 18 only, plus an adjuvant to boost the immune response.

The minimum age for enrolment in either trial was 15. Many girls become sexually active — and hence potentially exposed to HPV — in their mid-teens, so it is important to reach them beforehand. Younger girls were not enrolled because this would require longer trials to gain sufficient data to determine vaccination efficacy.

In both the Future and Patricia trials, participants were examined at least once each year for signs of HPV infection or epithelial lesions. Lesions are classified as either cervical intraepithelial neoplasia (CIN) grade 1, 2 or 3 (abnormal cell growth), or adenocarcinoma in situ (AIS) — a localized tumour that has yet to become invasive. Trial participants who developed CIN2 or higher-grade lesions (CIN2+) were treated, so their natural disease outcome is not known. However, the trials also differed in important ways.

The trials gathered data for four years after vaccination, although both Merck and GSK reported early results as soon as they had enough data to reach statistical significance. After an average of 3 years of follow-up, results for the two vaccines were similar: both were at least 98% effective in preventing CIN2+ associated with HPV types 16 or 18 (refs 1,2,3). Gardasil also had 100% efficacy against genital warts[2]. At this stage, the number of subjects diagnosed with CIN3 and AIS in any of the trials was too low to draw firm conclusions about the vaccines' efficacy on these measures, says Lehtinen. There were no serious side effects.

In the absence of a head-to-head trial, "it would be unfair to compare the two vaccines because the trials were not identical", says Jorma Paavonen, professor of obstetrics and gynaecology at the University of Helsinki, Finland, and medical investigator for the Finnish trials. Based on the initial efficacy and safety data, the US Food and Drug Administration (FDA) approved Gardasil in 2006 and Cervarix in 2009.

However, neither vaccine was able to clear established HPV infection; in other words, there was no therapeutic effect. From a public-health viewpoint, this reinforced the notion that the vaccines should be given to individuals before they become sexually active (although how long before — that is, the earliest age the vaccine can be given — has not yet been determined).

INTO THE REAL WORLD

Full results covering four years for all trial subjects have now been reported. The latest data, which have more statistical power, were published in 2010 for Gardasil[4,5] and 2011 for Cervarix[6]. So far, immunity has endured: antibody levels in the vaccinated women are as high as they were immediately post-vaccination. The final efficacy data against persistent infection with HPV types 16 and 18, and against premalignant lesions, confirm earlier findings (and are most impressive for women younger than 20). Furthermore, in an entirely separate trial in Costa Rica, Cervarix showed 84% efficacy against anal infection by HPV types 16 and 18 (ref. 7).

CORBIS

It is hoped that mass vaccination of girls will protect women and men from HPV-related cancers.

The near-complete protection conferred by both vaccines against infection with HPV types 16 and 18 "was an unexpected result — almost too good to be true", says Lehtinen. What's more, there was a surprisingly high level of effectiveness against HPV types not targeted by the vaccine — particularly with Cervarix, which seemed to protect against HPV types 31, 33 and 45. "The data regarding cross-protection were much stronger than expected — to everyone's delight," says Barbara Romanowski, clinical professor in infectious diseases at the University of Alberta in Edmonton, Canada, and principal investigator for the Canadian trials of Cervarix. But how these trial results translate into real-world protection against infection with non-vaccine types is yet to be determined, she adds, and monitoring is continuing.

As countries start mass HPV vaccination campaigns of girls, other effects might become apparent. Researchers in several countries, including Australia, the United Kingdom, the United States and some Scandinavian countries, are watching to see whether other HPV types begin to take over the niche previously occupied by types 16 and 18. A similar shift previously occurred with vaccines against pneumococcal infections, resulting in an increased circulation of pneumococcal serotype 19a, which the vaccine did not target. Such a shift in HPV could undermine any progress towards reducing cervical cancer cases. "We may see an overtaking of the ecological niche by non-vaccine types," Lehtinen warns, "but there is no evidence yet."

Researchers hope that mass vaccination will also lower the rate of infection in the unvaccinated population — in men, for example. Whether so-called herd immunity is achieved partly depends on the proportion of the population that receives the full vaccine dose (see 'Mass vaccination'). To this end vaccination for boys was recommended in the USA and Canada in November 2011, and plans to vaccinate 12- and 13-year-old boys in Australia from 2013 have also been announced.

Data on the impact of these population-based campaigns are beginning to trickle through, with Australia providing the first glimpse. More than 70% of eligible girls have completed all three courses, and there is already a marked decline in the prevalence of anogenital warts in both young women (59% fewer cases) and men (28% fewer)[8]. New data from cervical screening clinics in New South Wales and Victoria reveal that fewer young women have been infected with vaccine-related HPV types[9].

The state of Victoria has seen a decline of 0.38% in the incidence of high-grade (CIN2+) abnormalities in young women since vaccination began (number of new diagnoses within a 3-month period per 100 women tested)[10]. Perhaps more significantly, data for 2010 also reveal

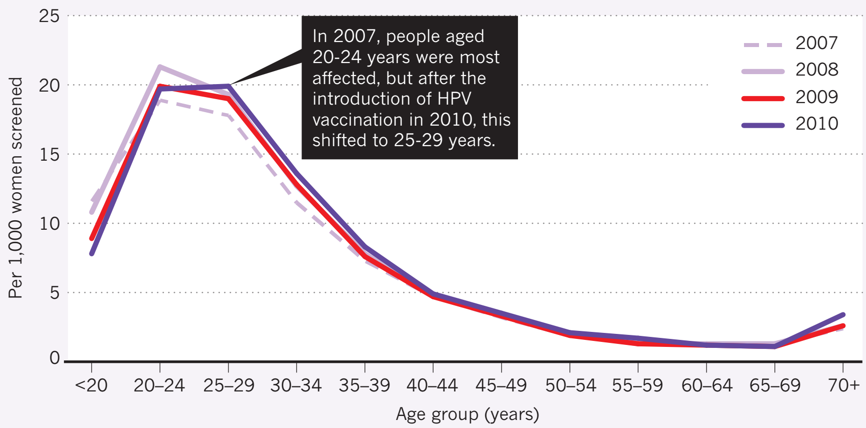
MASS VACCINATION

Few Western countries have achieved >50% coverage. For both vaccines, three doses are recommended, but several trials are investigating whether fewer doses are effective.

Country	Date vaccination campaign started	Uptake – at least 1 dose	Uptake – all 3 doses	Vaccine used
Australia	2007 (Gardasil), 2008 (Cervarix); (12–13 year olds)	74%	70.8% Range: 63.7% (Tasmania) to 79.6% (Australian Capital Territory) [11]	Gardasil and Cervarix
Canada	2007 to 2009, (9–14 year olds)	n/a	50% (Alberta and Manitoba) to 85% (Newfoundland, Nova Scotia and Quebec, for 2 or 3 doses.) [12]	Gardasil
Denmark	2009	80%	62% [ref. 13]	Gardasil
United Kingdom	2008, (12–13 year olds)	89%	83.8% [ref. 14]	Cervarix (changing to Gardasil in September 2012)
United States	2007, (13–17 year olds)	48.7%	32% [ref. 15]	Gardasil and Cervarix

HIGH-GRADE LESIONS IN AUSTRALIA

The number of women with high-grade abnormalities detected by histology screening dropped in women aged 20-24 years. Futher study will reveal whether this is the result of vaccination.



AUSTRALIAN DEPT. HEALTH AND AGEING.

that prevalence of CIN2+ abnormalities is now highest in the 25–29 age group, rather than in younger women. In women aged 20 to 24 years old — a group that contains the first girls to be immunized, including 14–18-year-olds on 'catch-up' immunization — the prevalence of high-grade lesions is "flattening out", says Julia Brotherton, epidemiologist and medical director of Australia's National HPV Vaccination Program Register in Carlton South (see graph).

Rather than rely on national averages, Australian researchers are in the process of linking cervical screening data to the national vaccination register to compare outcomes for individual women who received one, two or three doses against outcomes for unvaccinated women. "We hope by the end of this year to have more definitive results showing that it's specifically the vaccinated women who are getting most of the benefit," says Brotherton. Similar data for the United Kingdom and the United states are due out soon.

In Finland, by contrast, the success of the screening campaign in preventing cervical cancer has reduced the urgency to introduce the HPV vaccine. The National Institute for Health and Welfare has only recently recommended mass vaccination for girls, and the government has not yet given the campaign the go-ahead. In the meantime, researchers have an opportunity for a different kind of trial that would not be possible in countries where vaccination campaigns are already underway. Lehtinen and his colleagues are embarking on a new phase IV trial of Cervarix that will directly test the benefits of vaccinating boys as well as girls (see 'Vaccinate boys too', page S10). Depending on the results, "we could correct the decision to vaccinate only girls if we get data showing that vaccination of girls and boys is even better", says Lehtinen.

All these results are about preventing infection and abnormal cervical cell swabs. Data that might show efficacy against cervical cancer will take longer. "We'll need to wait for women who were vaccinated in their early teens to move through to their late twenties and onwards before we're truly able to see it," Brotherton adds.

So the long-term human study continues. Researchers are waiting to see whether vaccination leads to enduring immunity at the population level, and against which HPV types. But as Lehtinen says, the real test of the vaccines will be not in the data the clinical trials were designed to reveal. Ultimately, it's about whether the vaccine can prevent cancer and save lives. Those, he says, are "the most stringent end-points". ■

Julie Clayton *is a freelance journalist based in Bristol, UK.*

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REGISTERING LONG-TERM TRENDS

Clinical trials of the two HPV vaccines may have finished, but data are still accumulating.

Now the trials of Gardasil and Cervarix have formally ended, participants are back with their normal healthcare systems for routine cervical cancer screening and care. But those in Scandinavian countries such as Finland and Sweden can continue to provide information. These countries run national registries that routinely gather data on conditions such as autoimmune disease, pregnancy outcomes and cancer that are linked to personal identification numbers, helping researchers gather long-term vaccine efficacy data against cervical cancer and other HPV-related diseases. "Finland is a paradise of health registries," says Jorma Paavonen, professor of obstetrics and gynaecology at the University of Helsinki and medical investigator for the Finnish trials. Such a system will help illuminate the vaccines' long-term anticancer potential. "We can link vaccinated and unvaccinated individuals to the cancer registry to answer the question in a couple of years," he adds.

And they should have sufficient numbers. High interest levels meant that more girls volunteered for the Finnish trials than were needed. These girls now comprise an additional unvaccinated cohort of about 15,000 individuals. In the original trials, the control groups in Finland — 874 for Gardasil and 2,400 for Cervarix — were offered the vaccine once the trials were completed; approximately half accepted the offer, shrinking the control groups. In the new unvaccinated cohort, "we already have early results that there are a few cancer cases versus zero cases in the vaccine arm", Paavonen says. "The numbers are small, but the cases will accumulate rapidly."

The registries will also be useful in gathering data on adverse events. Despite a handful of media scares about their safety, "there's no evidence that these vaccines cause any harm", he adds. Nonetheless, he and other trial investigators are keen to continue monitoring at least for the next decade. ***J. C.***

VACCINATION

A durable design

Vaccines on the market aren't practical for the developing world — where cervical cancer hits hardest — but researchers are trying to make ones that are.

BY KATHARINE SANDERSON

Cervical cancer is most deadly in the developing world. According to the GAVI Alliance, a group of leading organizations working to improve access to immunization, 88% of the 275,008 women who died in 2008 from cervical cancer lived in developing countries.

At least 15 types of human papillomavirus (HPV) are implicated in cervical cancer. The two vaccines on the market, Merck's Gardasil and GlaxoSmithKline's Cervarix, offer protection against the most prevalent types, HPV 16 and HPV 18, which between them cause 70% of cervical cancer cases (see 'The global burden', page S2). But, for several reasons, these vaccines are not ideal.

Cost is an impediment. Susan Wang, a medical officer in the Expanded Programme for Immunization (EPI) at the World Health Organization (WHO) in Geneva, estimates that it costs between US$4 and $6 to vaccinate each girl, money that many developing countries can ill afford.

Developing countries also face huge logistical hurdles. Vaccines need cold storage to be viable, but this is difficult in regions with hot climates and unreliable electricity. Then there's the problem of ensuring the right people receive the right doses. Both vaccines require 3 injected doses over 6 months; in remote areas and in cultures where the need for vaccinations are poorly understood, this schedule is difficult to ensure.

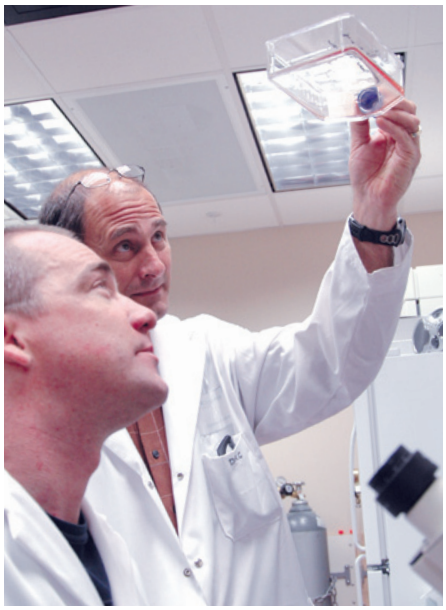
The current vaccines that target HPV 16/18 do not protect against 30% of cervical cancer cases. What's more, if HPV 16/18 are suppressed, there is a risk that other HPV types will proliferate in their place (see 'Trials of an anticancer jab', page S4). A vaccine offering broader protection against all cancer-causing strains is the objective.

What's more, prophylactic vaccines are useless for someone who is already infected. A therapeutic jab that could eliminate all HPV infection plus any cancerous cells would enable a far more effective vaccination programme in combating cervical cancer.

Next-generation HPV vaccines are in development. Scientists are targeting different proteins with the ultimate goal of developing a single-dose vaccine effective against more types of the virus, which is more durable and cheaper to make.

One strategy is to tweak the current generation of vaccines. Gardasil and Cervarix both use the L1 proteins that coat specific HPV types to make virus-like particles (VLPs). It is VLPs that trick the immune system into reacting to a viral intruder, triggering the production of antibodies that subsequently protect against HPV infection.

Gardasil includes four L1 proteins, Cervarix two. To broaden coverage, Merck is developing a vaccine that includes nine viral proteins. But this vaccine is likely to be expensive because it relies on existing, costly manufacturing methods. Virologist Lutz Gissmann from the German Cancer Research Centre in Heidelberg says that bombarding the body with so many antigen-provoking proteins can tire the immune system: there are instances (not in HPV) where adding an extra antigen to a vaccine lowers the immunogenicity of the others, he adds.



MS. SIAO YONG, DEELEY RESEARCH CENTRE, VICTORIA, CANADA

Darin Wick (foreground) and John Webb hold some HPV-infected tumour cells up to the light.

LOCAL PRODUCE

As an alternative approach, pathologist Richard Roden at Johns Hopkins University in Baltimore, Maryland, exploits another viral protein, L2. These L2 proteins stimulate a weaker immune response than L1 proteins, but one that is active against more types of HPV. Roden's group fuses together units of L2 from various HPV types to make a single polypeptide chain that can be expressed by *Escherichia coli* (not possible with L1 proteins). Roden contends that using bacteria to produce single polypeptides would simplify manufacturing to such an extent that the vaccine could be produced in local laboratories, bringing costs down significantly. The L2 vaccine is due to enter phase I clinical trials in early 2013. "This is fantastic," comments Gissmann, who is not involved with the work. "It's a completely different principle and I think it's doable."

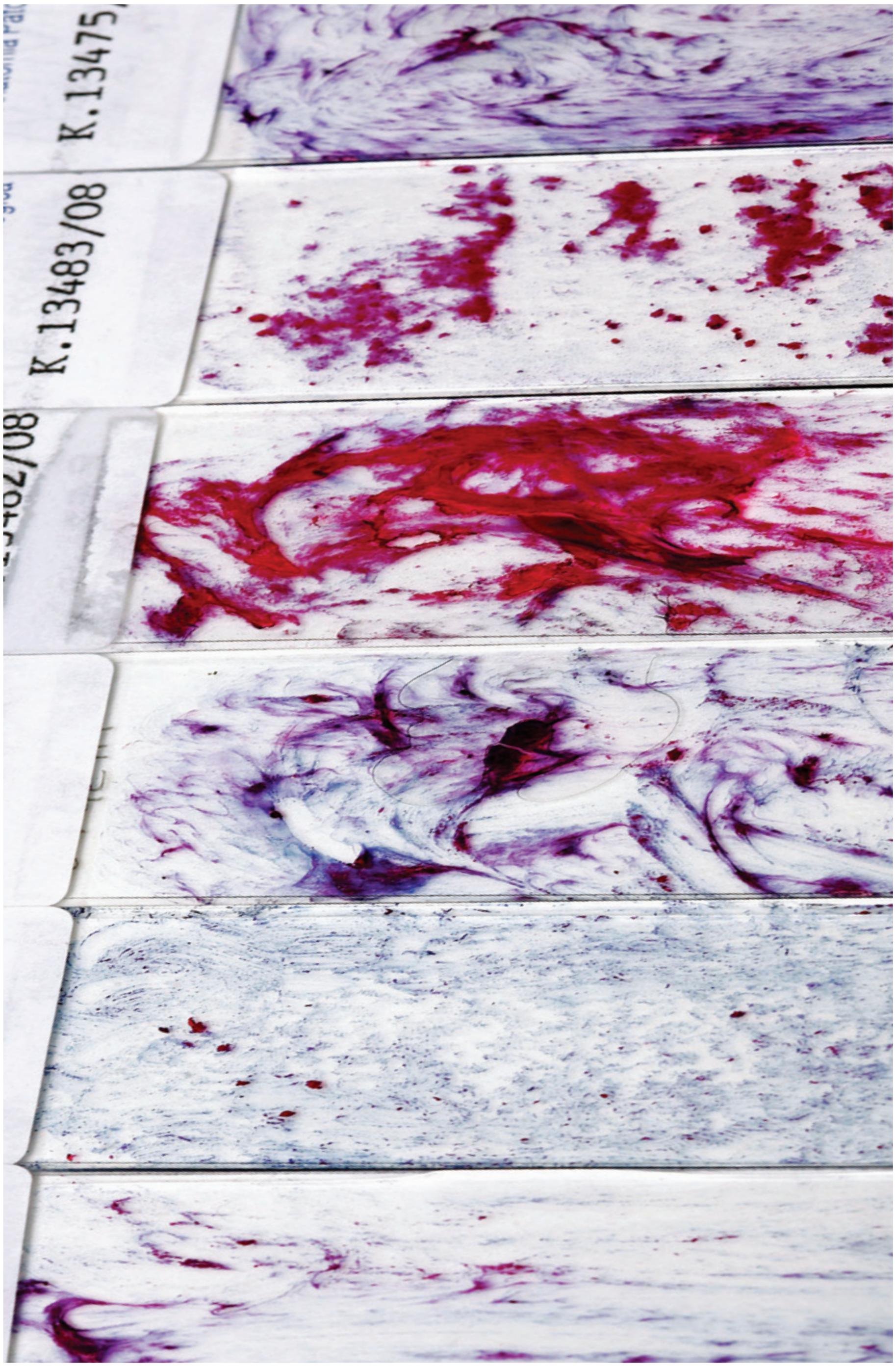
Other researchers are pursuing therapeutic angles. Immunologist John Webb at the BC Cancer Agency in Victoria, Canada, is developing a vaccine that targets E7 proteins. E proteins activate once HPV has infected a cell. They promote the growth of cancer cells by inactivating tumour suppressing proteins.

Antibodies are useless against E7 because, by this stage, the virus is inside the cell. Instead, an immune cell, $CD8^+$ cells, that kills virus-infected cells is the best form of defence. Past attempts to trigger CD8 responses have targeted particular disease-causing protein subunits. According to Webb, these techniques "work — but very inefficiently" because the subunits are so specific that the virus can easily evade the immune response. Webb's vaccine instead contains whole E7 proteins from the five most common cancer-causing HPV types, as well as poly(I:C) — a double-stranded RNA molecule that signals the presence of a virus. Together these molecules stimulate a swift build-up of CD8. In mice, administering the vaccine once a day for 4 days caused tumours to shrink — often disappearing — within 3 weeks (Wick, D. A. & Webb, J. R. *Vaccine* **29**, 7857–7866, 2011). Webb speculates that this concentrated exposure to two simultaneous agents helps the vaccine "emulate the effect of a true viral infection" and hence strengthens the result.

What's more, the whole-protein formulation of this vaccine, called Pentarix, makes it more stable than the minimal peptide approaches, and without the need for refrigeration, says Webb. "It may be better suited to developing-world settings than prophylactic vaccines."

Gissmann is optimistic that new vaccines will soon start to show success in clinical trials, solving some of the problems that have beset HPV vaccines on the market. So the next generation of HPV vaccines might finally be able to reach the people who need them most. ■

Katharine Sanderson *is a freelance journalist based in Toulouse, France.*



MAURO FERMARIELLO/SCIENCE PHOTO LIBRARY

Cells swabbed from the cervix are smeared on slides to spot abnormalities.

SCREENING

Testing times

Pap tests have been a mainstay of cervical cancer screening, but new tests, vaccines and knowledge might be changing that, including when and how frequently to test.

BY COURTNEY HUMPHRIES

Cervical cancer rates in developed countries have plummeted by as much as 70% since 1950 primarily thanks to intensive screening programmes using the Papanicolaou (Pap) smear test, which looks for abnormal cells of the cervical lining.

Pap tests have become a rite of passage for many women. And although screening programmes have been effective in countries that can afford to implement them, the tests are problematic. "Pap tests are notoriously insensitive," explains Eduardo Franco, a cancer epidemiologist at McGill University in Montreal, Canada. The tests only detect cancer in 50% to 75% of cases.

As cervical cancer develops slowly, repeating the test regularly can compensate for the tests insensitivity. But the frequency of testing (which has been annual in the United States and many countries, and every two or three years in others) Franco says, "is based on an admission that we have a very imperfect tool in our hand."

The role of Pap tests is changing, change driven by new tests to detect human papillomavirus (HPV) infection directly, as well as the wider recognition that the test can be too often invasive, unnecessary and a burden on women. HPV vaccination programs may help further scale back screening in the coming decades, and technologies may also help relieve the stark disparities in cervical cancer prevention worldwide. But medical organizations and governments are still struggling to identify the best approaches to deploy screening programs given the resources at their disposal.

NEW PARADIGMS FOR PREVENTION

The Pap test is a visible check for abnormal cells collected from the opening of the cervix during a pelvic exam and smeared on a glass slide. A newer method called liquid-based cytology, in which the swab is suspended in preservative fluid rather than smeared, produces more consistently useful cells. In either case, a technician looks for any abnormalities under a microscope. Women with cells showing signs of aberrant growth are referred for further examination and, if necessary, for treatment.

The past decade has seen the emergence of tests that detect HPV DNA in cervical swabs. Several versions of HPV tests are now on the market, including ones that detect any cancer causing varieties of HPV, as well as molecular genotyping assays that can specifically identify the two most carcinogenic types of the virus, HPV-16 and HPV-18. Research has found that these genotyping tests have 90-95% sensitivity; what's more the tests make it possible to intervene earlier because they can directly detect HPV infection before visible abnormalities manifest in cells.

Although HPV testing is more sensitive than Pap tests, they are not designed to identify the signs of cervical cancer.

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A cost analysis of DNA detection versus Pap tests:
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HPV infection is common, particularly in young women (approximately 45% of US women in their early twenties have been infected with HPV) and as many as 90% of people who test positive for HPV subsequently test negative within 6–24 months (see 'The global burden', page S2). A screening programme using HPV tests would identify too many young women who will not develop cervical cancer.

For older women, however, HPV testing has made a big difference. "It allows you to safely increase the screening interval," says Jack Cuzick, who heads the Centre for Epidemiology, Mathematics and Statistics at the Wolfson Institute in London. But how best to do that has been the subject of an evolving discussion. The United States has taken a conservative approach of adopting co-screening with Pap and HPV tests for women over 30. But studies in Canada and Europe have found that administering Pap tests only after a positive HPV test is more effective.

Many governments and medical organizations are starting to recommend delaying the onset of all screening; previous US guidelines, for instance, began screening at age 18 or within three years of starting sexual activity. Franco explains that in addition to the problems with testing young women for HPV, giving Pap tests to adolescent girls and young women can lead to misleading results — they often develop minor cervical abnormalities from HPV infections that rarely progress to cervical cancer and are not clinically important — and unnecessary treatments, some of which have been linked to miscarriage and complications with future pregnancies.

Different countries have different standards. In March 2012, consensus guidelines issued by the American Cancer Society, the American Society for Colposcopy and Cervical Pathology and the American Society for Clinical Pathology advocated not screening women before age 21, regardless of sexual history. The UK's national screening program tests at age 25; in Australia, the national screening program currently advises women to get Pap tests every 2 years from 18 years of age. Franco, who was involved in the US consensus guidelines, says that while research has shown that testing women between 21 and 24 years of age provides only an "infinitesimal" amount of protection, extending the age to 25 has stirred debate between those who favour an aggressive approach to cancer prevention and those who believe that testing should be minimized when possible.

ECLIPSED BY VACCINE?

As virtually all cases of cervical cancer are caused by HPV, it would seem that the availability of a vaccine that effectively prevents HPV infection would eliminate the need for cervical cancer screening. Most HPV vaccination programmes target girls 12–13 years old — well below screening age. "The population you're targeting with vaccines is different from screening," says Jane Kim, who performs cost-effectiveness analyses in cervical cancer care at the Harvard School of Public Health in Boston, Massachusetts. It will take decades before women who were too old to be including in vaccination programmes (usually set at age 26) pass the upper screening age of 65. Moreover, current HPV vaccines protect against only the most common cancer-causing strains of HPV.

Inadequate HPV vaccination programmes are also an issue. In the United States, for instance, only half of adolescent girls have received even one dose of the vaccine, with only 30% completing all three required doses (see 'Trials of an anticancer jab', page S4). Vaccine coverage needs to be a lot higher before it would be prudent to recommend reduced screening, says Mona Saraiya, a medical officer working in cancer prevention at the US Centers for Disease Control and Prevention in Atlanta, Georgia. The absence of a comprehensive system for tracking individuals' vaccine history is adding to the need for continued screening in the United States. Franco says that countries like the United Kingdom, Canada, and Australia, which have school-based vaccination programmes and better patient tracking, are better positioned to take advantage of vaccines and eventually develop separate screening policies for vaccinated and non-vaccinated women.

"If you're going to see women infrequently, you want to get a high rate of detection."

Researchers are trying to model the complexities of HPV infection and cervical cancer to better inform screening and vaccination policy. In Europe, a multinational study called PREHDICT could yield a statistical model to evaluate the cost effectiveness of prevention programs against HPV-related disease. One of the study's aims is to determine what would be the optimal way of combining screening and vaccination programmes given the health resources of a country, says its leader Johannes Berkhof, a biostatistician at VU University Medical Centre in Amsterdam. The study will also look at whether vaccination should be administered to women older than 26 years as well as to boys (who can pass HPV to women and develop HPV-related cancers and genital warts). To answer these questions, the multinational team is pooling resources such as data from longitudinal screening studies and vaccine clinical trials, and is amassing a database on the burden of HPV-related disease. The emphasis of the study will be on creating better models of the natural history of HPV infections — how they are acquired and cleared, and how natural immunity against a particular type develops — and on estimating the effects of vaccination on transmission rates.

In poor countries, cervical cancer is still a leading cause of cancer in women (see 'Prevention comes of age', page S11), in large part because governments cannot afford expensive screening programmes. HPV vaccination might be a cheaper alternative to screening programs. Kim says that it's important not to frame the issue as a choice between vaccination and screening. "The evidence for us has suggested that it would be cost effective to do both."

The question, then, is how to screen. The success of Pap testing in the developed world has depended on frequency of testing, which is more difficult to maintain in places that lack medical resources, says Kim. HPV testing, with its higher sensitivity, offers a way around this problem. "If you're going to see women infrequently, you want to get a high rate of detection." But HPV tests are more expensive than Pap smears, so Kim and other researchers have been modeling the cost-effectiveness of different screening strategies. In regions of the world with the highest cervical cancer incidence and mortality, such as East Africa, HPV testing may be worthwhile if done at longer intervals — like three to five times over a woman's life after the age of 30, says Kim.

Researchers are also trying to develop new ways to identify woman at risk for cervical cancer such as swabbing the cervix with diluted vinegar turning damaged tissue white. "It's a fairly low-cost, low-tech intervention," Kim says. Because the vinegar swab method doesn't require sending a sample to a laboratory, women can often be treated on the same day as the test — an important advantage for those who find it difficult to travel to a clinic.

THE FUTURE OF SCREENING

Cervical cancer screening programs may evolve as technologies develop. Recent innovations could lead to HPV tests that distinguish between specific high-risk strains. Pap tests may be further improved by adding cancer-specific markers to increase their sensitivity.

In countries that have followed the US's frequent screening model, Pap tests are seen as a ritual of preventive care. Lengthening the interval between tests represents a change for both women and doctors. Some doctors are concerned that screening less frequently will cause them to lose contact with patients, as well as increase the chance for legal liability, says Saraiya. But she adds that the uncoupling of Pap tests from annual preventive health exams might be easier to put in practice with younger patients free of these expectations.

New tests also require patient education. HPV tests put the emphasis on testing for a virus rather than cancer, with possible stigma and relationship stress that come with a sexually transmitted infection. "Women are used to Pap testing, but they haven't equated it with a sexually transmitted infection," says Saraiya. "It's important that we're crafting the correct counselling messages," she says, so that women continue to understand the long-term benefits of screening. ■

Courtney Humphries *is a freelance writer based in Boston, Massachusetts.*

PERSPECTIVE

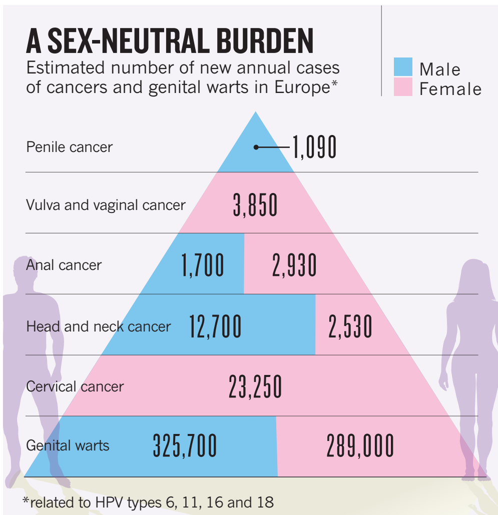
Vaccinate boys too

HPV-associated cancers in men are on the rise. By not vaccinating boys we are failing to gain maximum health benefit, argues **Margaret Stanley**.

Infection with human papillomavirus (HPV) is the cause of almost all cervical cancers, as well as a significant number of cancers of the vulva and vagina. These links make us think of HPV as a women's health problem. But that's not the case.

HPV is also the main cause of cancers of the anus, tonsils and tongue, and is a significant contributor to cancers of the penis, larynx, head and neck. It is estimated to be the causal agent in 5% of all human cancers[1]. HPV is also the cause of genital warts, which are the commonest sexually transmitted viral disease with a lifetime risk of acquisition at 10% (ref. 2). Despite the virus's health impact on both sexes, most countries' HPV immunization programmes are exclusively for females. Only the United States, Canada and Australia recommend vaccination for boys and men as well. Indeed, Australia has recently announced that 12- and 13-year-old boys will be vaccinated starting in 2013.

The rationale for these policies is straightforward. If a reduction in female cancer is the only public-health target, it is clear from mathematical models that male vaccination provides only a small added benefit. However, this approach fails to serve men who develop HPV-attributable cancers of the anus, penis, oral cavity and oropharynx, and who have an equivalent burden to women in terms of genital warts[2].



BEYOND THE CERVIX

Anal carcinoma is a rare cancer, but its incidence among men aged 20–49 is on the rise. Rates of anal cancer are highest in men who have sex with men (MSM); the incidence in this group is estimated to be equivalent to that of cervical cancer in an unscreened population, and is even higher in HIV-infected MSM[3].

Rates of HPV-associated cancers of the head and neck, known as oropharyngeal squamous cell carcinomas (OSCCs), have increased dramatically in both men and women in developed countries over the past 20–30 years. In the United States, incidence of HPV-related OSCC is higher in men than women, as is the prevalence of oral HPV infection. Unless preventive measures are put in place, the annual number of HPV-positive OSCC cases in the United States is predicted to exceed that of cervical cancer by 2020 (ref. 4).

The good news is that HPV vaccine trials have shown efficacy against HPV infection and related anogenital disease in men. It is less clear whether vaccination of males to prevent HPV infection is worth the expense. Most older economic models conclude that when female vaccination coverage is high, vaccination of boys is not cost effective[5]. Indeed, once female vaccination coverage exceeds 50%, 'herd immunity' will eventually develop, and men who have sex only with women will be protected. Unfortunately, few countries have achieved the required rate of female HPV vaccination. Furthermore, MSM in this scenario receive little or no benefit from herd immunity and remain vulnerable to preventable HPV-associated disease. In the cost-effectiveness models, MSM represent too small a fraction of the population to justify general male vaccination. But not all national immunization programmes meet strict cost-effectiveness criteria. Vaccination against meningococcal infection in children is not cost effective, for example, but society accepts it because the prevention of such a serious disease is a worthwhile public-health goal.

TARGETING MEN

One way to make a male HPV vaccine programme cost effective would be to target MSM exclusively[6]. But such a limited vaccination policy would raise important issues of access, equality and ethics. It would also lengthen the time needed to establish herd immunity, and until that time, unvaccinated men will continue to develop preventable HPV-associated cancers. Anyone implementing a strategy targeting MSM would face the tricky question of what is the best age for such a vaccine. For optimal vaccine impact, MSM would need to be reached in early adolescence, before they start having sex. But questioning boys in this age group about their sexual orientation would produce highly unreliable results, in large part because orientation is not yet firmly established. (Such questioning would also be likely to elicit parental outrage.) But the alternative — targeting men in their late teens — would lower the vaccine's effectiveness because this age group includes large numbers of sexually active men who have had ample occasion for HPV exposure. Moreover, requiring the disclosure of sexual orientation as a prerequisite for immunization is ethically questionable.

In many developed countries, including those in western Europe, the burden of HPV-associated cancers in men is now comparable to that in women[2]. Cervical cancer, the dominant HPV-related cancer in women, can be prevented through vaccination and screening, but there is no screening for anal cancer or OSCC — serious diseases that tend to occur in younger age groups than cervical cancer, present at a later stage with associated mortality, and show significant morbidity with impaired quality of life after therapy. All men, regardless of sexual orientation, face a significant and increasing risk of HPV-associated disease. It is not ethical, fair or socially responsible to have a public-health policy that forces men to rely on herd immunity, which won't be reached for decades. Let's start vaccinating men now. ■

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COURTESY OF MERCK

As half a dozen African countries roll-out national HPV vaccine programmes, these schoolgirls read why they need the vaccine.

PUBLIC HEALTH

Prevention comes of age

Sub-Saharan countries lag behind in screening and treatment for human papillomavirus. But national efforts and the introduction of low-tech methods could change that.

BY MICHAEL EISENSTEIN

It wends its way across bumpy terrain packed in coolers loaded in the back of trucks, lashed to bicycles or even carried by hand. But however it gets there, the human papillomavirus (HPV) vaccine, a key weapon in the fight against cervical cancer, is finally making its way to African communities that desperately need it.

In the developed world, access to diagnostic resources, highly trained physicians and modern medical technology has diminished the threat of cervical cancer. The UK death rate from this disease fell by 70% between 1970 and 2008[1], and national vaccination programmes promise further decreases. But the picture remains bleak in the developing world, particularly in sub-Saharan Africa. In eastern Africa, for example, cervical cancer incidence and mortality rates are respectively 5-fold and 12-fold greater than in Western Europe.

In much of Africa, the problem is exacerbated by the equally high prevalence of HIV infection, which weakens women's immune defences and allows HPV to rapidly achieve the chronic infection state that is the prelude to cancer. "Because of HIV/AIDS, we began to see cancer of the cervix appearing in very young women," says Mulindi Mwanahamuntu, a gynaecologist at the University Teaching Hospital in Lusaka, Zambia. "In some cases, we're finding it at the age of 20." Subsidized antiretroviral drugs have extended the lives of many HIV-infected patients, but the persistence of HPV means that many coinfected women will instead succumb to cervical cancer. "We will see a huge spike in cervical cancer in the next 10 years or so, largely driven by HIV," says Lynette Denny, professor of obstetrics and gynaecology at the University of Cape Town in South Africa.

CHEAP SHOTS HAVE BIG IMPACT

In the United States, a full course of Merck's Gardasil or GlaxoSmithKline's Cervarix vaccine costs more than US$300 — far beyond the reach of low- and middle-income countries. However, both companies have made sizeable donations to support early-stage vaccination programmes in low-income countries, with promises of price subsidies in the future. A donation of two million doses of Gardasil over three years has allowed the government of Rwanda to embark on Africa's first national HPV vaccination programme[1], expected to reach 130,000 girls in 2012. Lesotho followed suit in January with a national vaccination programme of its own, and the Nigerian government recently announced its intention to launch nationwide HPV immunization by the end of this year.

Meanwhile, half-a-dozen other African countries are pushing towards full-scale national vaccination. European and Tanzanian physicians have joined forces to determine the efficacy and costs of school-based vaccination programmes, for example. According to Deborah Watson-Jones, an epidemiologist at the London School of Hygiene & Tropical Medicine, the programme was made possible by donations from Merck's Gardasil Access Program and the US non-profit organization Axios Healthcare Development. "After discussions with the Ministry of Health, it was agreed that we would apply for that donation for Tanzania and do a school-based vaccination programme in three districts around Mwanza city, including urban and rural areas," she says. The demonstration was a success, delivering the full course of three shots to about three-quarters of the targeted girls.


IMAGES COURTESY OF PATH

Schoolgirls at Ibanda Kyarukumba Primary School in Uganda wait their turn for the HPV jab. In Uganda, projects have reached up to 90% of target populations.

Funds from the Bill & Melinda Gates Foundation enabled the non-profit health organization PATH to conduct what many consider to be a seminal demonstration programme of HPV vaccination in Vietnam, Peru, India and Uganda. In each country, PATH ensured that national governments and local communities had a central role in designing vaccine delivery procedures to ensure maximum participation and to make it easy to rapidly expand the programmes. "We talked to parents and women's groups and community leaders and advocates and district health officials, all the way up the chain to the national level, to get an understanding of what people knew and what information they needed to accept the vaccine," says Scott LaMontagne, country research manager for PATH's HPV vaccine project. The programmes proved a remarkable success, routinely vaccinating more than 80% of the target population, reaching more than 90% in some communities[2].

The systematic approach adopted by PATH has guided many of the efforts that followed. According to Emmanuel Mugisha, PATH's vaccination project manager in Uganda, education and good communication were critical. "Even among health professionals, knowledge is limited — especially about the link between HPV and cervical cancer," says Mugisha. Health workers also confronted the association of HPV infection with poor hygiene or prostitution, and removing this stigma was critical to boosting participation. Mugisha describes a lengthy, multipronged outreach process, combining poster and leaflet campaigns, radio phone-ins, and town-hall meetings led by PATH-trained health workers from the local community. Most parents also sought additional advice. "There was a process of talking with the neighbours or aunties or other family members, or seeking further information from health workers, or even going online," says LaMontagne.

Schools offered an ideal venue for reaching most adolescent girls in a given community, but there were many problems. For example, most HPV vaccination programmes target girls within a specific age range, but this can be problematic in regions where birth records are patchy or non-existent and where families do not closely track calendar age. "Both Uganda and Tanzania have experienced similar challenges with age-based delivery," says Watson-Jones. "Age literacy is quite poor in some parts of Africa, and people may guess or lie about age to get their children into school." Furthermore, the wide scatter of ages within a given primary school could send health workers scrambling to find 10-to-12-year-old girls, disrupting teachers' work and making tracking a challenge. "Targeting a specific class or grade is much more feasible," says Mugisha. "If every girl in [a particular year at school] is supposed to be vaccinated, then follow-up becomes very easy even if they change schools."

Although fewer doses may confer some protection, full vaccine efficacy requires a full course of three doses — a regimen that makes scheduling a complicated chore. Absenteeism was a primary factor in reducing overall vaccine coverage, and planners also had to contend with individual school schedules for holidays and exams, and, in some cases, differences in term structure. With insufficient resources to hire additional personnel just for HPV vaccination, health workers' time must be planned and managed carefully before dispatching them, sometimes across considerable distances, to deliver the vaccine. This effort requires close coordination with schools, and teachers and administrators were often an important ally. "They were instrumental to the success of the programme," says Mugisha.

HANDLING HIDDEN COSTS

Both Tanzania and Uganda are looking to scale up vaccination by adapting lessons learned during their demonstration programmes. "In Tanzania, they're going for a class-based approach with health-centre back-up for girls who miss a dose," says Watson-Jones, "and the programme will vaccinate a younger class because we're slightly worried about primary-school dropouts." Thanks to donations from GlaxoSmithKline and Merck, Mugisha estimates that Uganda has vaccinated more than 30,000 girls since launching its demonstration programme in 2008, and the government is now discussing how to vaccinate on a national scale.

Efforts to scale up vaccination will benefit from the support of the GAVI Alliance, based in Geneva, Switzerland. This organization, which underwrites vaccine access for many of the world's poorest nations, including 38 countries in Africa, announced in April 2012 that it will add the HPV vaccine to its portfolio. "All in all, almost 60 countries will have national introduction in the next few years," says LaMontagne, "and these numbers will only increase with the commitment by GAVI to support this vaccine."

But many programme costs will not be covered by GAVI funding. Some of these are start-up costs and will subside over time. For example, the initially high investment in education and outreach will become less prominent as vaccine messaging takes hold in communities. On the other hand, the cost of basic supplies such as syringes will remain steady. Furthermore, HPV vaccination is logistically challenging, as it cannot be bundled with existing health interventions, which are typically targeted at infants or young children. "There are generally no platforms in place for the administration of vaccine to pubescent or adolescent girls," says Denny. In Uganda, PATH attempted to piggyback on an existing programme called Child Days Plus, which provides nutritional and anti-parasitic interventions to schoolchildren. Vaccine coverage was limited by the use of an age-based approach, but Mugisha believes this programme still offers the best opportunity. "Health workers are already going

COURTESY OF JHPIEGO

to the schools," he says. "You're basically just adding the HPV vaccine to their package, and that eliminates additional transport costs."

The GAVI Alliance is helping governments get started by implementing a separate funding programme to support the launch of vaccine demonstration schemes. PATH, meanwhile, is sharing its experiences and the training materials it has developed to date. Rwanda consulted PATH while designing its national programme, for example, and LaMontagne hopes that other nations with leaner budgets could avoid some expense by drawing on lessons learned by PATH. "There's experience to be used," he says, "and no need for countries to think they have to start their planning from scratch."

SPOTLIGHT ON A QUIET KILLER

To defeat cervical cancer, vaccination programmes must be coupled with robust screening and treatment programmes (see 'Screen and treat'). This in turn requires political will, as the cost of sustaining any large-scale effort to control cervical cancer will ultimately fall to national governments. However, with limited resources available for a seemingly endless roster of public-health problems, including malaria, tuberculosis and HIV/AIDS, advocates for preventing cervical cancer must shout to be heard. "We're getting a lot of requests for information and for technical assistance," says Ricky Lu, a cervical cancer specialist at Jhpiego, a non-profit public-health organization associated with Johns Hopkins University in Baltimore, Maryland. "But ministries of health have limited resources to address many competing priorities."

An odd silence seems to surround this killer of women, which has tragically come to be associated in some settings with immorality or irresponsible sexual behaviour. "We have women who are champions about breast cancer because they are survivors and they are able to talk about it, but it's very rare you hear somebody talking about being a cervical cancer survivor," says Mwanahamuntu. "This stigma exists everywhere, and it needs to stop."

Fortunately, some of Africa's most powerful women have taken notice. The Forum of African First Ladies Against Breast & Cervical Cancer has become a forceful voice for raising awareness, organizing conferences and bringing together medical experts and policymakers to take action. As more nations pledge their commitment to fight cervical cancer, there may be cause for hope that this disease is finally getting the attention it deserves. "Women need to be empowered to control their own sexuality and reproductive health," says Denny. ■

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SCREEN AND TREAT

First responders

In Côte d'Ivoire, Tsigue Pleah (right) demonstrates cryotherapy to a local health worker.

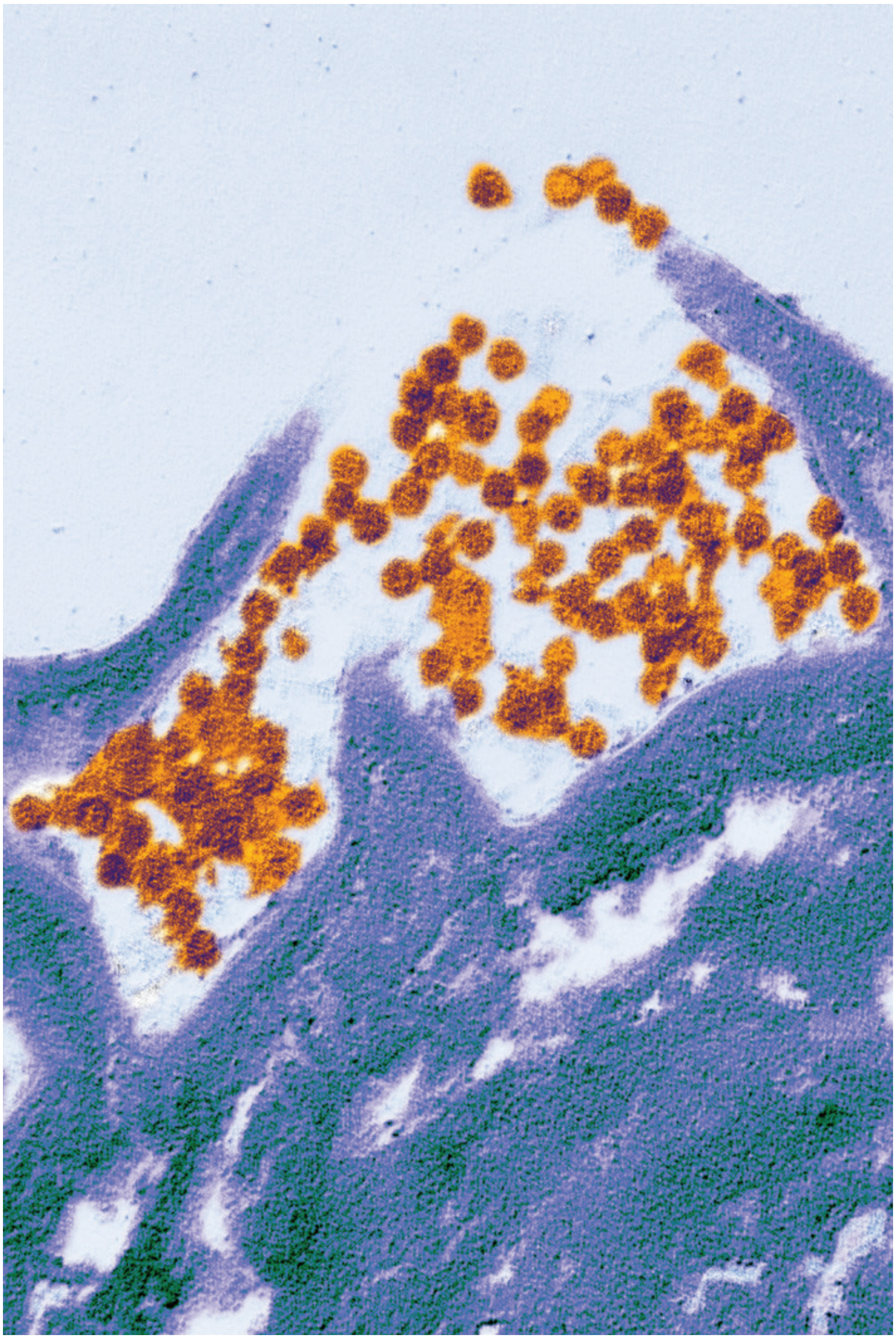
Vaccines are a vital tool in the prevention of cervical cancer, but they're not enough. Even if every adolescent girl were vaccinated, women already exposed to HPV would remain at risk. "We're mopping the floor," says gynaecologist Mulindi Mwanahamuntu of the University Teaching Hospital in Lusaka, Zambia. "But the tap is still running."

The lack of options makes it critical to diagnose cervical cancer quickly. However, most poor nations lack resources for routine Pap smear screening. As an alternative, organizations such as Jhpiego, a non-profit health organization affiliated with Johns Hopkins University in Baltimore, Maryland, are promoting a low-cost alternative: visual inspection with acetic acid (VIA). Swabbing the cervix with an acetic acid solution, such as vinegar, turns precancerous lesions white, allowing health workers to recognize and remove them in the same visit. Removal is typically achieved by cryotherapy, using compressed carbon dioxide to cool a probe to temperatures that kill and remove precancerous cells with minimal side effects. "The cure rate for lesions of the right size — meaning less than 75% of the surface of the cervix — is around 90%," says Ricky Lu, a cervical cancer specialist at Jhpiego. Lu adds that this single-visit 'screen and treat' approach is particularly valuable in poor areas of Africa where it is difficult to persuade women to return for a follow-up.

Jhpiego is helping the governments of Kenya, Tanzania, Mozambique, Ivory Coast and Burkina Faso to develop and implement national cervical cancer prevention programmes. One of the biggest success stories unfolded under the auspices of the Cervical Cancer Prevention Program in Zambia (CCPPZ), which trains Zambian health workers to perform VIA and cryotherapy. After the implementation of VIA, and the training of health workers to perform it, screening has blossomed. "Our core team has screened over 80,000 women within the last five years," says Mwanahamuntu, a CCPPZ director. "Before that, we barely screened 5,000 people in about 40 years."

When properly performed by well-trained staff, VIA achieves specificity — the percentage of negative test results that represent true negatives — that rivals or surpasses the Pap smear[3] (see 'Testing times', page S8). However, the test is prone to false-positives. "It would never pass an FDA audit," says obstetrician and gynaecologist Lynette Denny of the University of Cape Town in South Africa. But as many women in poorer nations may be screened just once in a lifetime, a tendency towards over-detection is probably better than the reverse.

The infrastructure built for VIA-based programmes can be used for other technologies when they come along. Women in high-income nations have access to kits that detect DNA from tumorigenic HPV strains, but these are expensive. However, there are high hopes for careHPV, a highly accurate test to detect HPV DNA, developed at PATH and commercialized by Qiagen, based in Hilden, Germany. This test can provide far more accurate test results than VIA in about 90 minutes. Although careHPV is too expensive to replace VIA, Qiagen has made clear its intention to make it affordable. The company has already donated 250,000 careHPV kits to Rwanda's national cervical cancer programme, and imminent commercial launches in India and China could fuel broader demand and lower future costs. As a result, state-of-the-art diagnostics could come within reach of far more people. ***M.E.***



DR KLAUS BOLLER/SCIENCE PHOTO LIBRARY

The surface of a human wart (purple) shedding human papmillomavirus particles (brown), that can take root on another area of skin or on another person.

PATHOLOGY

Three questions

Linking specific types of HPV with cervical cancer and developing effective vaccines against should be celebrated. But there are gaps in our understanding of these viruses and how they cause disease.

BY LAURA VARGAS-PARADA

It is extremely common but has a set of peculiar features. To complete its vicious cycle of infection, human papillomavirus (HPV) has developed tricks to evade and even regulate the body's immune system. Alpha-HPVs, a genus that contains the two most dangerous HPV types, frequently infect the mucosal epithelial cells lining the surface of the cervix (see 'The global burden', page S2). Hidden inside a keratinocyte — the most common epithelial cell — HPV can remain almost invisible to the surveillance of the immune system.

This 'hitchhiker' stratagem has also hindered scientists' quest to fully understand how HPV triggers the development of cervical cancer. "The difficulties we face stem from the lifecycle of the virus," says Margaret Stanley, a virologist and epithelial biologist at the University of Cambridge, UK. This lifecycle, she explains, "is played out as the epithelium differentiates". Studying this process, she adds, "is technically very challenging because our *in-vitro* systems may not reproduce the *in-vivo* environment". The lifecycle of HPV can be viewed as a dance between the virus and the keratinocyte: in an infected keratinocyte, HPV gene expression is spatially and temporally regulated in an intricate, step-by-step performance.

Despite HPV's efforts to remain hidden, infection seems to be transient in most women. "More than 80% of all sexually active women will have seen HPV at least once, and fewer than 1% have actual problems with such an infection," says Sjoerd H. van der Burg, immunologist and head of the Experimental Cancer Immunology and Therapy Group at Leiden University Medical Center in the Netherlands. "This must mean that in most women an effective immune response is launched."

But this observation raises a major unanswered question.

1. Why do only some women develop disease?
Most women infected with HPV do not develop cervical cancer. But it remains a mystery why the immune response varies so much from individual to individual. Why do only some people develop disease? "I would rephrase the question," says van der Burg, " to what is it that does not trigger effective immunity in some patients while most others respond?" In a series of clinical studies, van der Burg's group found that women who remain healthy tend to have "well-balanced T-cell immunity" against several antigens produced by the virus. By contrast, people who develop persistent infections lack this response — their immune systems fail to recognize viral antigens[1].

T cells are part of the adaptive immune response, which springs into action once the innate immune system has done its work. But

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it is still not clear, says van der Burg, whether the response comes from $CD8^+$ T cells, which destroy HPV-infected cells, or by $CD4^+$ T cells, which help other white blood cells fight infection. This question is crucial to fully understanding how viral immunity develops.

Also unknown is how HPV escapes from the keratinocyte. Discovery of the molecular pathways that govern these processes "will probably lead to identification of genetic differences in these pathways, and hopefully to an understanding why people fail to respond appropriately", says van der Burg.

For Patti Gravitt, a molecular epidemiologist with Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland, the key question to understanding the natural history of HPV is more fundamental.

2. Does the immune response clear HPV infection?

Screening methods to determine whether someone is infected with HPV are based on detecting viral DNA in cervical swabs. "We always assumed that the presence of viral DNA in a cervical swab is equivalent to being infected; if we don't see the viral DNA then you are not infected," says Gravitt, who developed a way to detect and genotype the DNA of HPV while working at Pleasanton, California-based Roche Molecular Systems.

But this oversimplifies the situation. Although the absence of viral DNA suggests that the virus has been cleared, it could also mean that the virus has entered an undetectable persistent state known as latency. "Current assays have these methodological limitations," says Xavier Castellsagué, a cancer epidemiologist at the Catalan Institute of Oncology (ICO) in Barcelona, Spain, and director of the WHO/ICO Information Centre on HPV and Cervical Cancer. "Even if we detect some HPV DNA, we cannot tell whether it is just an inert piece of the virus or a potentially active infection," he says. "We're at the limit of HPV DNA detection." To find the answer, he explains, researchers will have to "follow up to detect persistence and early lesion development".

In most cases, a viral infection will trigger an effective immune response that clears all traces of the virus from the body. But some viruses evade the immune response. The HIV and hepatitis B viruspersist by a constant level of viral replication. Other viruses, such as the herpes simplex viruses, become latent in an apparently disease-free stage that can, under certain conditions, reactivate into a productive infection.

One current hypothesis is that HPV is not cleared from the body but remains in a latent state, although firm evidence for this idea is lacking. "We are working on HPV latency but are not there yet," says Ciaran B. J. Woodman, a cancer epidemiologist at the Cancer Research UK Institute for Cancer Studies at the University of Birmingham, UK.

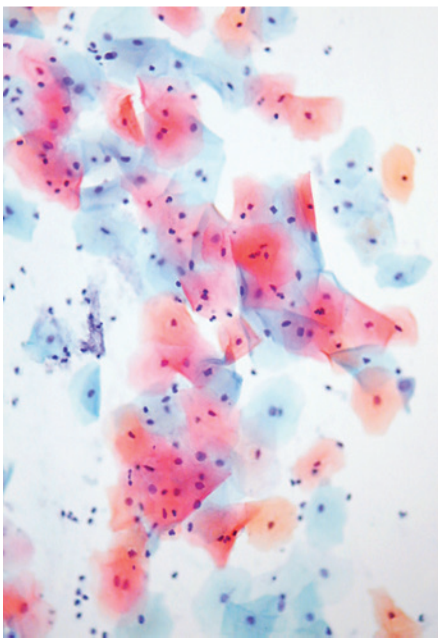
No mechanism for latency has yet been

"There are consequences to not knowing the answer to these questions."

established, and its impact on otherwise healthy women is unclear. "If you are immunocompetent, you probably have very sporadic bouts of viral replication that your immune system immediately recognizes and brings under control," says Gravitt.

Clinical observations and studies in severely immune-suppressed women, as well as in women receiving organ transplantation, seem to support this idea[2]. For now, however, technological limitations in human studies make it difficult to demonstrate HPV latency and reactivation, Gravitt says, but experimental evidence from animal models of papillomavirus infection shows that latency is occurring.

HPV could have more than one way of being latent, says John Doorbar, a molecular virologist at the National Institute for Medical Research in London. Doorbar theorizes that HPV could survive either as a persistent, asymptomatic infection or as a latent genome



HPV-infected cells with clear cytoplasm and enlarged nuclei (purple).

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whose replication is controlled by the host's immune system.

In 2011, Doorbar's team studied latency in a rabbit model of papillomavirus infection. By dissecting the tissue with a laser, the researchers were able to investigate the process in unprecedented detail[3]. The group's findings "prove that papillomaviruses can persist in the site of infection after lesion clearance and that their genomes are found in the basal layer [of the mucosa] with little evidence of productive infection in the layers above", says Doorbar. Although he cautions that human papillomaviruses may not necessarily behave the same way, "for the moment we assume that they do".

But while researchers seek to pinpoint how HPV survives in the body, treating women at risk of cervical cancer raises an important question about the screening mechanism.

3. How can women at risk of HPV-related disease be identified?

Developing a good screening test requires knowledge of how long latency can last, and how differences in the nature of the infected cell can affect disease outcome. To solve those puzzles, scientists are trying to identify biomarkers for disease progression. Viral load and HPV type have both been suggested to predict the risk of developing cancer. But so far, says Woodman, robust clinical markers are lacking.

For example, certain strains of HPV are responsible for almost all the cases of malignant disease. Being infected with one of these high-risk types of HPV may be necessary for cervical cancer, but it is not enough on its own[4]. "Prognostic tests are needed to identify individuals who are at risk of progression," says Doorbar. His group is studying how gene expression patterns change in a defined way during disease progression. Such patterns, he says, will help to identify different stages of disease and help to assess the risk of progression.

A recent study, published in June 2012, identified a specific population of cells located in a particular area of the cervix where most, if not all, HPV-associated cancers arise[5]. The presence of such cells might therefore be the marker that scientists have been seeking to differentiate between benign and precancerous lesions.

On the whole, HPV has seen several successes for medical research. A virus was identified as the cause of a serious type of cancer (see 'On the case', page S16). As a result, specific screening methods were devised, and a highly effective prophylactic vaccine was developed (see 'Trial of an anticancer jab', page S4). These are rare events in public health.

But the gaps in current knowledge nevertheless provide serious barriers to further progress. "There are consequences to not knowing the answers to these questions," says Gravitt. Indeed, until the holes in the fabric of understanding are patched up, she says, it will be difficult to know "who needs to be screened, who is at risk, what a positive test result means, and who needs to be vaccinated. But the perception is that we have the problem solved." ■

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COURTESY OF THE DEUTSCHES KREBSFORSCHUNGSZENTRUM, HEIDELBERG.

Q&A Harald zur Hausen

On the case

A Nobel prizewinner for pinning cervical cancer on human papillomavirus, Harald zur Hausen still investigates viruses. Nature Outlook *talks to the medical doctor–turned–virologist about other possible culprits.*

What first raised your interest in HPV?
I was only interested in cervical cancer. I picked up on papillomaviruses by looking into the literature from the 1930s: researchers such as Richard Shope and Peyton Rous had investigated lentil-like structures on wild US cottontail rabbits. They found that taking extracts from these lesions and infecting domestic rabbits produced similar warts that converted gradually to malignant tumours. I also found a few anecdotal reports that genital warts in humans occasionally converted into malignant tumours. These findings triggered the idea that there may be an agent in the genital lesions that could also cause cervical cancer; perhaps this agent has a different cancer-causing potential in the cervical mucosa than in the external genital skin.

Since I had seen papillomavirus particles in genital warts, I thought HPV would be a good candidate. However, as there are few particles in genital warts, it took us a long time to characterize and isolate HPV type 6 and it was very disappointing not to find HPV-6 in cervical cancer samples. We kept looking, however, and a year later we found a related virus in genital warts: HPV-11. Using HPV-11 as a probe, we finally managed to isolate the distantly related HPV-16 and HPV-18 and link them to cancer. It was not a Eureka moment.

What are the prospects for cancer prevention?
There is a good chance of drastically reducing cervical cancer by vaccination. In Australia, where there is more than 70% coverage, the data are clear cut — the precursor lesions are being prevented. There's no evidence yet that cancer is being prevented, as this takes time. The average latency is 15–25 years; since the vaccine is 6 years old, we still have a 10–20-year wait. But look at hepatitis B. In Taiwan where there is compulsory vaccination since 1984, the rate of liver cancer has dropped by 70%. Something similar will happen with cervical cancer.

Can we eliminate HPV?
HPV-16 and -18 could probably be eliminated if we have a global programme. You could theoretically achieve this by vaccinating only girls, but you would need very high coverage. I'm a strong advocate for vaccinating boys as well: we'll reach the goal much faster by vaccinating both sexes. The disadvantage is that the cost is very high.

How common is HPV?
HPV is part of an enormously heterogeneous family of viruses with more than 200 individual genotypes. Virtually every part of our skin is infected by papillomaviruses, and some prevent apoptosis (cell death), cutaneous types in particular. If skin cells survive after mild damage by ultraviolet radiation, this might provide some protection. I suggested recently that we examine white and black skin to see whether there are differences in the viral load and to determine whether there is an evolutionary advantage in having large amounts of cutaneous HPVs. But there is another side to blocking apoptosis: if the cell sustains too much damage but doesn't die, it could turn cancerous.

There is an old suspicion that HPV is involved in non-melanoma skin cancer. For many cancers, if the number of cases increases in line with imunosuppression, an infectious agent might be involved. In breast cancer, for example, immune suppression might have a protective effect that we don't yet understand. In the majority of skin cancers, there is fewer than one copy of HPV DNA per cell, which suggests the virus is not a major player.

Could microbes lie at the root of more cancers?
Based on epidemiological characteristics, it is very likely, particularly in some leukaemias and lymphomas. At the moment, less than 10% of the total leukaemia/lymphoma burden is attributable to viruses or bacteria.

Colon cancer is interesting. If you look at its geographic incidence, it is linked to areas with high red-meat consumption. For example, in most regions in India, where beef is not consumed, it is a rare form of cancer. The only exception is Mongolia where they have low rates of colon cancer and eat a lot of red meat, but there they eat yak. It's not too far fetched to think there might be a cow-specific infectious agent.

We are trying to identify agents in cattle and in humans. There is evidence that some exist, but we don't know whether they are carcinogenic or not. These are anelloviruses, specifically torque teno viruses (TTVs). They have some interesting properties: micro-TTVs, for instance, are totally rearranged tiny molecules that replicate autonomously — why and how, we don't understand. In addition, there are chimaeric forms, consisting of TTV DNA and host-cell sequences derived, in part, from growth-promoting genes — some of which are known to be important in the development of cancer. ■

Interview by **Michelle Grayson**, *senior editor for* Nature Outlook *in London.*

GYNECOLOGICAL CANCER

More evidence supporting human papillomavirus testing

Philip E. Castle

Clinical trials have consistently demonstrated the superior sensitivity of human papillomavirus (HPV) testing compared with cytology (Pap) testing for identifying women at risk of cervical cancer. Rijkaart *et al.* have now shown that adding HPV testing to routine cervical cancer screening can further reduce the risk of cervical cancer compared to Pap testing alone.

Castle, P. E. *Nat. Rev. Clin. Oncol.* **9**, 131–132 (2012); published online 14 February 2012; doi:10.1038/nrclinonc.2012.16

Cervical cancer is the third most common cancer and cause of cancer-related mortality in women. Where cytology (Pap)-based programs have been launched effectively, cervical cancer rates have been reduced by 50–90%. It is now widely accepted that persistent cervical infections by approximately 13 genotypes of human papillomavirus (HPV) cause virtually all cases of cervical cancer and its immediate precursor—a precancerous lesion also known as cervical intraepithelial neoplasia grade 3 (CIN3). The identification of HPV as the necessary cause of cervical cancer has led to two technical advances in the prevention of this malignancy: first, prophylactic HPV vaccination for primary prevention of HPV infection and second, HPV DNA testing for screening to detect CIN3 for secondary prevention. Rijkaart *et al.*[1] have now reported the results of the POpulation-BAsed SCreening study AMsterdam (POBASCAM), a large randomized clinical trial that included nearly 45,000 women aged 29–56 years who were already participating in routine cervical cancer screening.

> "...HPV testing can and will improve cervical cancer screening"

The aim of the study was to evaluate the co-testing of HPV DNA and cytology (intervention arm) versus routine cytology screening alone (control arm).[1] Women were randomly assigned 1:1 to either the intervention or control screening—with approximately 20,000 eligible, consenting women in each group—and were re-screened 5 years later. Those women in both arms who had severe cytologic abnormalities were referred immediately for colposcopy, whereas women with equivocal or mildly abnormal cytology, or with a HPV-positive test, were followed up over the next 18 months. After this period, women with evidence of cytologic abnormalities and/or viral persistence were also referred to colposcopy. The key findings of the study were: first, co-testing was more sensitive than cytology alone for detection of CIN2 (equivocal precancer) and CIN3 in the first round of screening, which resulted in reductions in CIN3 and, more importantly, cancer in the second round of screening (which included co-testing in both arms) 5 years later; second, negative results in the co-testing group in the first round of screening were more reassuring than negative cytology against a diagnosis of CIN3 and cancer in the second round of screening; third, among those women in the co-testing group, negative cytology added very little additional reassurance against a diagnosis of CIN3 and cancer in the second round of screening compared with HPV testing alone; and fourth, detection of HPV16 (the most carcinogenic HPV genotype) in the co-testing group was a very strong predictor of CIN3 and a diagnosis of cancer, especially in the first round of screening.

The POBASCAM study confirms the findings of several large randomized trials in Europe,[2,3] North America,[4,5] and India,[6] showing the superior sensitivity of HPV testing compared with cytology alone. In the larger studies that included a follow up, the increased sensitivity of HPV testing compared with cytology for precancerous lesions translated directly into greater safety following a negative test, and a detectable reduction in cancer in the next round of screening.

Practice point

Adding human papillomavirus testing to cytology reduces the incidence of cervical cancer and provides superior safety following a negative test compared with cytology alone.

The added safety of including HPV testing into routine cervical cancer screening can be used in two ways to benefit patients. First, screening intervals can be extended, reducing the harm associated with screening without exceeding the acceptable cancer risk established by the preceding cytology-based screening program. This tradeoff of interval and cancer risk is well illustrated by a pooled analysis of European studies, which showed that the 2-year risk of CIN3 or more-severe diagnosis (CIN3+) following a negative cytology was approximately equal to the 6-year risk of CIN3+ following a negative HPV test.[7] Another observational study has also shown that the 3-year risk of CIN3 or CIN3+ following a negative cytology was approximately equal to the 5-year risk of CIN3+ following a negative HPV test.[8]

> "The shift emphasizes the strengths of the two tests when done sequentially"

A second way to benefit patients is by reducing the cancer risk for a given screening interval, as shown by Rijkaart *et al.*[1] Thus, despite the high-performance cytology program already implemented in the Netherlands that was 86% as sensitive as the HPV testing as illustrated by the POBASCAM trial, adding HPV testing to this cytology program will potentially reduce the incidence of cervical cancer.

The data from the POBASCAM[1] trial also illustrate that there is little added benefit of co-testing versus screening with HPV testing alone. In the first round of screening, 4.7% of CIN3+ and 5.8% of CIN2+ were diagnosed in women with HPV-negative, cytology-positive co-tests. The 5-year risk after a HPV-negative, cytology-negative co-test was not appreciably different than the 5-year risk after a negative HPV test (0.205% versus 0.206%, respectively); one case of CIN3 was diagnosed following a HPV-negative, borderline or mild dyskaryosis cytology at the second round of screening.

The annual rates in the Netherlands for cervical cancer incidence and mortality are some of the lowest in the world. However,

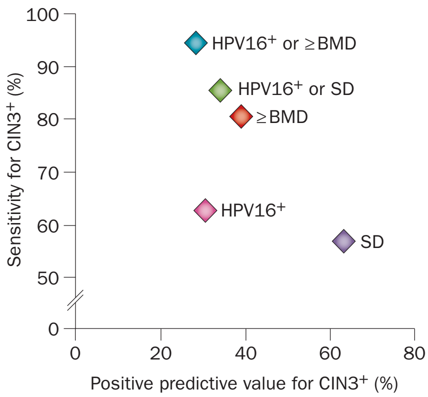

Figure 1 | Sensitivity versus positive predictive value for CIN3 or more severe diagnoses (CIN3+) for combinations of cytologic interpretation and HPV16 detection among HPV-positive women. Data are from Rijkaart *et al.*[1] Abbreviations: BMD, borderline or mild dyskaryosis; ≥BMD, borderline, mild, or more severe dyskaryosis; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; SD, severe dyskaryosis.

despite the very successful and efficient cytology program that screens women starting at the age of 30 years every 5 years until the age of 60 years, as a direct result of the POBASCAM study, the Netherlands is implementing a change in their national screening program from only cytology to primary HPV testing every 5 years starting in 2013. In the new HPV-based screening program, women with an HPV-positive test will undergo cytology testing at 0 and 6 months. Any abnormal Pap test result on either round will lead to an immediate referral to colposcopy. Women with a HPV-positive test and with a normal Pap test result on their return after 6 months will be rescreened again in 4.5 years.

In a worldwide context, the introduction of HPV testing into routine cervical cancer screening leads to a paradigm shift, by reducing routine screening cytology currently carried out on the entire population to only those women who have tested positive for a necessary cause of cervical cancer, HPV (corresponding to 5–15% of the population). The shift emphasizes the strengths of the two tests when done sequentially. HPV testing provides a good screen for the general population to rule out disease in healthy women, provided that clinicians do not intervene on the basis of only a positive HPV result (with the notable exception of a once or twice-a-lifetime screen-and-treat program designed for resource-limited regions). HPV testing is reliable, can be fully automated, and can achieve high volumes of testing per unit time. Adding cytology to an HPV test for general screening provides very little benefit to the patient but significantly inflates screening costs.

As a screening approach complementary to HPV, cytology is more specific for CIN3+, and provides useful risk stratification among women who are HPV positive—that is, it helps distinguish HPV associated with CIN3+ versus benign HPV infections that are destined to be cleared without medical intervention. For example, moderate and severe dysplasia cytology, the equivalent of high-grade squamous intraepithelial lesion cytology in The Bethesda System for classification of cervical cytology, is a very specific marker for underlying CIN3+, and women with these indications need immediate and completed follow up.

In the USA, where there is no national screening program, or in other countries with poor follow-up systems for women who are HPV positive with normal cytology,[9] it may be desirable to increase the sensitivity of CIN3+ on the initial screen. As shown in this study,[1] detection of HPV16, or HPV16 and HPV18, may identify women with a HPV-positive, cytology-negative test who are at high absolute risk of CIN3+. In POBASCAM, women with HPV-positive, cytology-negative test who tested positive for HPV16 were at a significantly higher risk of CIN3+ than women who tested negative for HPV16 in round one of screening (11.3% versus 1.5%, respectively, $P<0.0001$ [Fisher's exact test]) and in round two (8.6% versus 1.9%, respectively, $P=0.02$ [Fisher's exact test]). Results from testing for HPV16, or HPV16 and HPV18, can be combined with data from cytology in different ways to achieve the desired or optimized performance,[5] as illustrated in Figure 1. In the future, promising new biomarkers, such as INK4, HPV E6 and/or E7, if validated may be used to complement, enhance, or even replace cytology.

In conclusion, the POBASCAM trial adds to the growing body of evidence that HPV testing can and will improve cervical cancer screening. Recently, the US Preventive Services Task Force (USPSTF) judged that there was insufficient evidence to recommend HPV testing for cervical cancer screening.[10] This conclusion was reached in part because the randomized controlled trials conducted in Europe and elsewhere did not evaluate exactly how HPV testing would be used in the USA.[1–7] However, a synthesis of the data from the randomized controlled trials and large observation studies,[8] plus the knowledge of the well-defined natural history of HPV and cervical cancer, leads to an inevitable and rational conclusion that, when used correctly, HPV testing can efficiently and cost-effectively benefit women at risk of cervical cancer, whether living in The Netherlands, USA, or any other high-resource or lower-resource country.

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Acknowledgments
The views expressed represent only those of the author and do represent the views of The American Society for Clinical Pathology.

Competing interests
P. E. Castle declares associations with the following companies: Merck Sharp & Dohme, Roche. See the article online for full details of the relationships.

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British Journal of Cancer (2011) 105, 337 – 339

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Clinical Studies

Self-sampling and HPV testing or ordinary Pap-smear in women not regularly attending screening: a randomised study

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BACKGROUND: Most women with cervical cancer have not participated in Pap-smear screening. Self-sampling of vaginal fluid in combination with high-risk HPV testing may be a method to increase the attendance rate.

METHODS: A total of 4060 women, 39 – 60 years old, who had not attended the organised Pap-smear screening for 6 years or more were randomised into two equal groups. A study group was offered to self-sample vaginal fluid (Qvintip) at home and/or recommended to attend the Pap-smear screening. The collected fluid after self-sampling was examined for the presence of high-risk HPV (Hybrid Capture 2 method). Controls were only recommended to attend the Pap-smear screening. The end point was a histological identification of CIN2 – 3.

RESULTS: The participation rate was 39% (771 out of 2000) in the self-sampling group and 9% (188 out of 2060) in the conventional cytology ($P < 0.001$). The number of histological CIN2 – 3 alterations detected was 0.4% (8 out of 2000) among women offered self-sampling of vaginal fluid and 0.07% (3 out of 4060) in women offered Pap-smears. The odds ratio (OR) for offering self-sampling and HPV testing instead of Pap-smear screening for detection of CIN2 – 3 was OR = 5.42 (95% CI: 1.30 – 31.8).

CONCLUSION: Offering self-sampling of vaginal fluid followed by a high-risk HPV test was considerably more effective for detection of histological CIN2 – 3 lesions in comparison with offering Pap-test in a midwife reception in women not regularly attending organised screening.
British Journal of Cancer (2011) **105,** 337 – 339. doi:10.1038/bjc.2011.236 www.bjcancer.com
Published online 5 July 2011

Keywords: cervix; human papilloma virus; self-sampling; cytological screening

For documentation of the efficiency of organised Pap-smear screening for cervical cancer, nationwide audits are valuable. An investigation in Sweden showed that the majority of women (65%) with cervical cancer had not attended the organised Pap-smear screening, and around 25% developed cancer despite a regular participation due to the occurrence 'false-negative' smears (Andrae *et al*, 2008). A number of 'false-negative' smears indicates a low sensitivity of Pap-smear screening. A recent study, using primary screening with a high-risk HPV test for identification of histological CIN2 – 3 lesions, showed that the sensitivity of a single Pap-smear to detect CIN2 – 3 alterations is around 50%, also indicating a low sensitivity of cytological screening (Ronco *et al*, 2010).

To increase the participation rate and decrease the incidence of cervical cancers in the County of Uppsala, women not attending organised Pap-smear screening were, since the year 2006, offered self-sampling of vaginal fluid at home in combination with a high-risk HPV test (Stenvall *et al*, 2006; Sanner *et al*, 2009). Initially, during 2006 – 2007, a pilot study comprising 600 women not attending Pap-smear screening was performed (Stenvall *et al*, 2006). These women were not included in the present investigation. The HPV-positive women are recommended a follow-up in a gynaecological surgery (colposcopy clinic) or a midwife reception (family planning clinic). Preliminary results indicate that the self-sampling method is an attractive alternative for women who choose not to participate in the organised screening. Around 40% of women not regularly attending a midwife reception for smear sampling accept the home sampling method and most women attending a midwife reception for smear collection would prefer to collect vaginal fluid at home, if they were offered that possibility (Wikström *et al*, 2007a). In addition, the risk for obtaining 'false-negative' results is minimal (Ronco *et al*, 2010).

In this study, women not regularly attending in the organised Pap-smear screening programme were randomised into two groups of equal size. One group was offered ordinary Pap-smear screening and the other group the possibility of self-sampling of vaginal fluid at home as an alternative to Pap-smear screening. The main outcome measures of the study were the attendance rate and the identification of histological CIN2 – 3 lesions in both groups.

MATERIALS AND METHODS

A total of 4060 women, 39 – 60 years old, who had not participated in the organised Pap-smear screening for 6 years or more were collected from the local data base at the Department of Pathology and Cytology, Uppsala University Hospital, Sweden in January 2007.

The women were randomly divided into a study group of 2000 women and a control group of 2060 women. In the study group, all women were offered self-sampling at home with a self-sampling

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Received 24 February 2011; revised 24 May 2011; accepted 31 May 2011; published online 5 July 2011

device (Qvintip, Aprovix AB, Uppsala, Sweden) as an adjunct to organised Pap-smear screening. In the control group, women were only advised to participate in Pap-smear screening. In the organised Pap-smear screening, women 25–60 years old are invited for smear sampling every third year. Women, who choose not to attend receives additional invitation letters once a year.

All 2000 women in the study group were sent an information letter by post, and after a few days, they received the self-sampling device, instructions how to perform the sampling of vaginal fluid and to send the collected material to our laboratory in the enclosed, prepaid return letter. The procedure was free of charge and the women were also reminded in a second invitation. At the laboratory, the samples were used for high-risk HPV testing with the Hybrid Capture 2 (hc2) method (Qiagen AB, Solna, Sweden). The HPV test identifies 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The hc2 method can detect HPV DNA concentrations over 1 $pg\,ml^{-1}$, which is proportional to the light emission of the positive control and corresponds to 5000 HPV genomes per specimen in the well. The results of the HPV test were mailed to all women participating in the study. All information on women in the study group was collected in the database of the department, together with records on the Pap-smear screening and histopathological examinations.

The women who were high-risk HPV positive were recommended a follow-up examination at a midwife reception or a gynaecological surgery. In women examined by a gynaecologist, a biopsy from the cervix was obtained, whereas in women attending a midwife reception a repeated cytology, often in combination with a cervical sample for HPV analysis, was taken. All women offered self-sampling at home were also offered to participate in the organised Pap-smear screening.

The women in the control group were invited to a midwife reception for collection of cervical smear, within the framework of the organised screening programme. Women with ASCUS or CIN1 alterations observed in the screening were called for re-examination at the midwife reception and women with CIN2–3 cell changes were admitted to a gynaecological reception for colposcopy and cervical biopsy. The women paid 100 SEK (around 10 EUR) only for the first Pap-smear collection.

At the end of December 2007, all women who had performed self-sampling of vaginal smear at home in combination with a high-risk HPV test, and all women in the study and control groups participating in the Pap-smear screening were identified. Women who were HPV positive or showed abnormal cytology (ASCUS–CIN3) were followed until December 2009. The end point of the study was a histopathological CIN2–3 lesions observed in cervical biopsies or after cervical cone resection.

RESULTS

In the study group, 679 out of 2000 women (34%) accepted to perform self-sampling of vaginal fluid at home and send the collected material to our laboratory for high-risk HPV analysis, whereas 100 out of 2000 women (5%) preferred to attend a midwife reception for Pap-smear sampling. In total, 779 out of 2000 women (39%) in the study group participated in the screening. Among the controls, 188 out of 2060 women (9%) attended the Pap-smear screening programme. The difference in attendance rate between the two groups was strongly significant ($P<0.001$; Table 1).

A high-risk HPV-positive reaction was recorded in 41 out of 679 women (6.0%) performing self-sampling. The prevalence of HPV infection decreased with age, it was 7.5% (23 out of 305) in women 39–49 years old and 4.8% (18 out of 374) in women aged 50–60 years.

Of HPV-positive women, 24 out of 41 (59%) visited a gynaecological surgery, directly or via a midwife reception, for further examination including colposcopy and biopsy within 1–7 months after the collection of vaginal fluid. A total of 16 out of 41 (39%) only visited a midwife reception. The compliance of the HPV-positive women was 98% (40 out of 41). One woman had moved out of the county and was not possible to reach.

Table 1 Recruitment of 39- to 60-year-old women not attending organised cytological screening for more than 6 years by invitation for self-sampling of vaginal smear at home and/or Pap-smear screening (study group, 2000 cases) and offering only Pap-smear screening (control group, 2060 cases)

Categories	Study group	Controls
Total number of women	2000	2060
Self-sampling of vaginal fluid	679	0
Pap-smear screening	100	188
Not attending	1221	1872
Total number of participating women	779/2000 (39%)	188/2060 (9.1%)*

*$P<0.001$.

Table 2 Light microscopic morphology in 24 cervical biopsies of high-risk human papilloma virus-positive women

Light microscopy	Number of cases
Normal	12/24 (50 %)
CIN1	4/24 (17%)
CIN2–3	8/24 (33%)[a]

[a]One case with only cytological findings of CIN3.

The biopsies showed normal histology in 12 cases, CIN1 in 4 cases and CIN2–3 in 8 cases (Table 2). Women with CIN2–3 lesions were treated with cervical cone resection, whereas women with CIN1 and 4 women with normal histology and a persistent HPV-positive reaction were offered a continuous follow-up. Of the 16 women visiting only a midwife reception, 1 was HPV positive and 1 showed ASCUS. They were followed up, and in no case, a histological CIN2–3 was recorded.

A total of 100 women in the study group and 188 of the controls participated in the Pap-smear screening. Of these women, one cytological slide was not representative, one showed ASCUS, one CIN1 and three CIN2–3. The women with ASCUS were HPV negative, the women with CIN1 had a normal biopsy and the women with CIN2–3 were operated with cone resection, which histologically verified the CIN2–3 lesions.

All 4060 women in the investigation were offered collection of Pap-smear at a midwife reception, and 288 chose to participate. Of the participating women, three were identified with histological CIN2–3 on the cervix. A total of 2000 women were offered self-sampling of vaginal fluid at home in combination with HPV testing, and of 679 participating women, 8 had histological CIN2–3 lesions on the cervix. The odds ratio (OR) for identification of histological CIN2–3 lesions with the self-sampling at home method in comparison with Pap-smear screening was OR = 5.42 (95% CI: 1.3–31.8; Table 3).

DISCUSSION

As mentioned in the introduction, non-attendance is the major problem in countries with an organised Pap-smear screening for cervical cancer (Bos *et al*, 2006; Andrae *et al*, 2008; Lindqvist *et al*, 2008). Although this fact has been known for many years, no simple method to increase the coverage to the cytological screening is available (Eaker *et al*, 2004; Oscarsson *et al*, 2008). However, combinations of different invitations are reported to increase the number of detected precursor lesions (Eaker *et al*, 2004).

The method of self-sampling of vaginal fluid at home seems to be an attractive alternative for the non-participating women. It is less time consuming and it offers a possibility to avoid the

Table 3 Number of women with histological CIN2–3 lesions among women offered self-sampling of vaginal smear at home in combination with high-risk human papilloma virus testing and in women offered organised Pap-smear screening

Categories	Self-sampling of vaginal fluid	Pap-smear
Total number of women	2000	4060
Number of participating women	679/2000 (34%)	288/4060 (7.1%)
Number of women with CIN2–3	8/679 (1.2%)[a]	3/288 (1.0%)
Number of CIN2–3/total number of women	8/2000 (0.4%)[b]	3/4060 (0.07%)[b]

[a]One case with only cytological findings of CIN3. [b]Odds ratio = 5.42 (95% confidence interval: 1.30–31.8).

gynaecological chair position. The HPV test is also about twice as sensitive as a Pap test to identify histological CIN2–3 lesions. Several studies have shown that 30–50% of women not attending midwife receptions for Pap-smear screening accept the self-sampling method (Stenvall *et al*, 2006; Sanner *et al*, 2009; Gök *et al*, 2010). In this study, 87% of the attending women preferred self-sampling when they had the possibility to choose between self-sampling and Pap-smear screening. The fact that the self-sampling alternative was free of charge has probably contributed to the high attendance rate in this group. However, among women visiting a gynaecological surgery, 90% explained that they would prefer self-sampling at home instead of Pap-smear collection at a midwife reception provided that this possibility was available (Wikström *et al*, 2007a).

Although there is a general interest in increasing the coverage of the organised Pap-smear screening, there are also some objections against the use of HPV tests in organised screening for cervical cancer. One main reason is that, despite the high sensitivity, the HPV test is considered to have a too low specificity (Wright *et al*, 2004; Meijer *et al*, 2009). A number of women will be identified with high-risk HPV infection but without any cytological alterations, and no method is available for treatment of the HPV infection.

However, it must be kept in mind that the prevalence of high-risk HPV infections decreases with age and in post-menopausal women HPV infections are almost as uncommon as cell alterations (ASCUS–CIN3) in the Pap-smear screening. Consequently, in women 50 years and older HPV tests are almost as specific as Pap-smear screening (Wikström *et al*, 2007b; Sanner *et al*, 2009). Furthermore, the sensitivity of conventional cytology decreases markedly in post-menopausal women and most women with CIN2–3 lesions on the cervix display normal cytology (Gustafsson *et al*, 1995; Gyllensten *et al*, 2010). For that reason, self-sampling of vaginal fluid at home and HPV testing seems to be a reliable method to increase the attendance rate and also the sensitivity of the screening in countries with an organised Pap-smear screening programme. It is also indicated that self-sampling and HPV testing may be an attractive method for screening of menopausal women, an age category in which only around 20% of the maximal effect of Pap-smear screening remains and in which most women with cell alterations (ASCUS–CIN3) are high-risk HPV negative (Gyllensten *et al*, 2010).

A power analysis was performed. The number of participating women was however limited by the financial support for the study. The study population is too small to document differences in sensitivity between HPV and Pap-smear screening but it shows that self-sampling at home is an alternative to ordinary repeated calling of Pap-smear collection to non-responders.

ACKNOWLEDGEMENTS

The study was supported by the County Council of Uppsala and by the Medical Faculty of Uppsala University. The study was evaluated by the local ethic committee (Dnr2004:M-202 and 2009/001). Professor Pär Sparén, Department of Medical Epidemiology, Karolinska Institute, is acknowledged for randomisation of the material.

Conflict of interest

Erik Wilander is a minority shareholder in the company Aprovix AB, marketing Qvintip. All the other authors declare no conflict of interest.

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TIMELINE

Why do viruses cause cancer? Highlights of the first century of human tumour virology

Patrick S. Moore and Yuan Chang

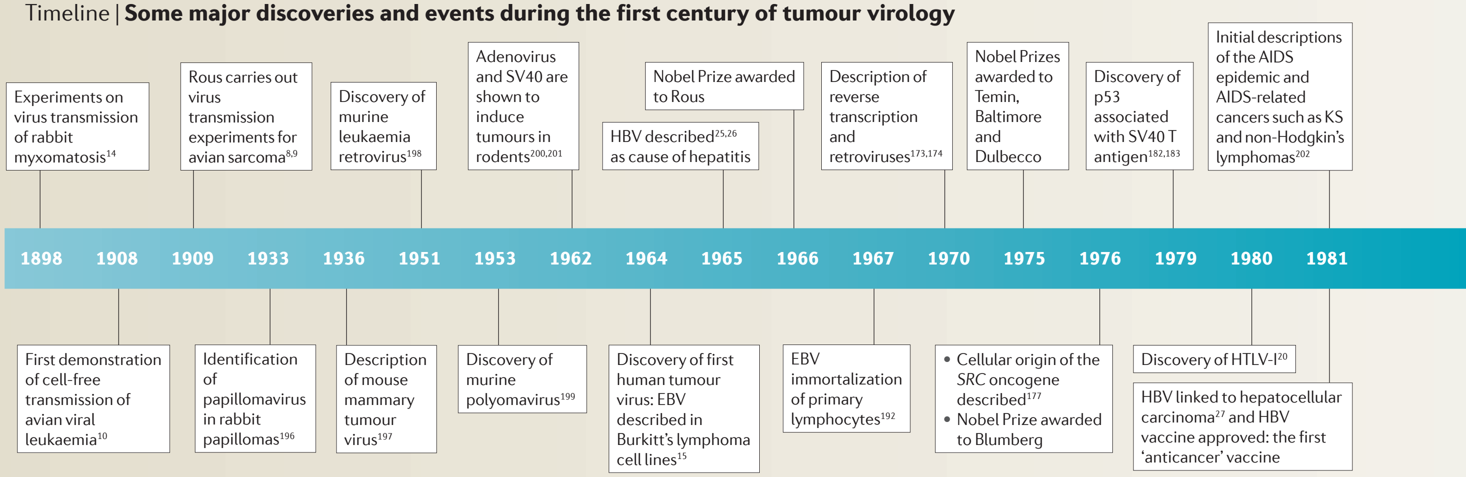
Abstract | The year 2011 marks the centenary of Francis Peyton Rous's landmark experiments on an avian cancer virus. Since then, seven human viruses have been found to cause 10–15% of human cancers worldwide. Viruses have been central to modern cancer research and provide profound insights into both infectious and non-infectious cancer causes. This diverse group of viruses reveals unexpected connections between innate immunity, immune sensors and tumour suppressor signalling that control both viral infection and cancer. This Timeline article describes common features of human tumour viruses and discusses how new technologies can be used to identify infectious causes of cancer.

The burden of viral infections in cancer is high but underappreciated by much of the cancer research community. The International Agency for Research on Cancer estimates that one in five cancer cases worldwide are caused by infection, with most caused by viruses[1,2]. These cancers are particular public health problems for the developing world, as well as for underserved and immunosuppressed populations in developed countries. Most importantly, these cancers have readily identifiable targets for diagnosis, prevention and therapy. Vaccination programmes against two human tumour viruses, hepatitis B virus (HBV) and human papillomavirus (HPV), have already begun to alter age-old cancer patterns on an international scale[3–5]. In this Timeline article, we provide an overview of the major milestones in research on viruses and human cancer (TIMELINE) and highlight common features among the human cancer viruses (TABLE 1). Non-viral infectious causes for human cancer are reviewed elsewhere[6,7].

Discovery of tumour viruses

On 1 October 1909, Francis Peyton Rous began his famous cancer virus transmission experiments at the Rockefeller Institute, USA, on a 15-month-old barred Plymouth Rock hen that had been brought to him by a farmer from Long Island, New York, USA. The chicken had a sarcomatous chest tumour that Rous successfully transplanted into other chickens that were related to the same brood[8,9]. By 1911, he had shown that the cancer could be transmitted through cell-free tumour extracts and thus must be caused by a small transmissible agent, probably a virus. These experiments knowingly built on the pioneering work of two Danish scientists, Vilhelm Ellerman and Oluf Bang (FIG. 1), who published similar results in 1908 on the viral transmission of avian erythroblastosis[10]. As cancers are not contagious, viral causes for chicken cancer were shortly thereafter relegated to being scientific curiosities[11]. Rous gave up his studies on viral cancers 4 years later and little further progress was made in tumour virology until the 1930s when mammalian tumour viruses began to be described[12,13]. Rous eventually returned to viral tumour biology through his studies with Richard Shope on the cottontail rabbit papillomavirus in 1934. These studies led to investigations of the co-carcinogenic effects of coal tars on virus-induced tumours. Interest in viral causes for cancer redoubled in the early 1950s following Ludwik Gross's discovery of an acutely transforming murine retrovirus (BOX 1) and a polyomavirus that caused murine tumours[14]. The culmination of this first century of tumour virology would be celebrated with Nobel Prizes awarded in 2008 for the discovery by Harald zur Hausen of high-risk HPV strains that cause cervical cancer and the discovery of HIV, an agent that does not initiate cancer but indirectly 'sets the stage' for malignancy through immunosuppression, by François Barré-Sinoussi and Luc Montagnier.

Timeline | **Some major discoveries and events during the first century of tumour virology**

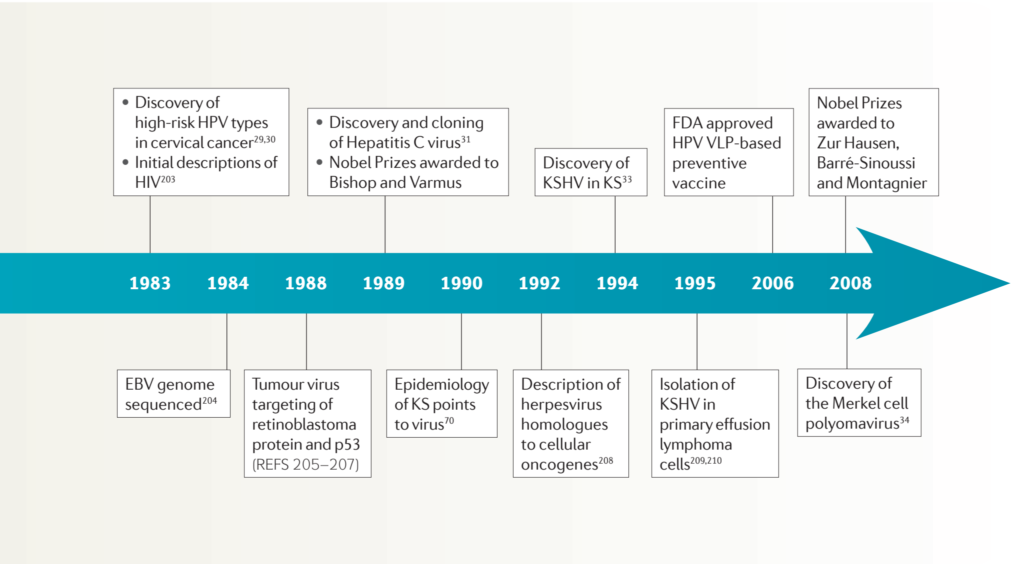


EBV, Epstein–Barr virus; FDA, US Food and Drug Administration; HBV, hepatitis B virus; HPV, human papillomavirus; HTLV-I, human T-lymphotropic virus-I; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma herpesvirus also known as human herpesvirus 8 (HHV8); VLP, virus-like particles.

Table 1 | **The human cancer viruses**

Virus	Genome	Notable cancers	Year first described	Refs
Epstein–Barr virus (EBV; also known as human herpesvirus 4 (HHV4))	Double-stranded DNA herpesvirus	Most Burkitt's lymphoma and nasopharyngeal carcinoma, most lymphoproliferative disorders, some Hodgkin's disease, some non-Hodgkin's lymphoma and some gastrointestinal lymphoma	1964	15
Hepatitis B virus (HBV)	Single-stranded and double-stranded DNA hepadenovirus	Some hepatocellular carcinoma	1965	25
Human T-lymphotropic virus-I (HTLV-I)	Positive-strand, single-stranded RNA retrovirus	Adult T cell leukaemia	1980	20
High-risk human papillomaviruses (HPV) 16 and HPV 18 (some other α-HPV types are also carcinogens)	Double-stranded DNA papillomavirus	Most cervical cancer and penile cancers and some other anogenital and head and neck cancers	1983–1984	29, 30
Hepatitis C virus (HCV)	Positive-strand, single-stranded RNA flavivirus	Some hepatocellular carcinoma and some lymphomas	1989	31
Kaposi's sarcoma herpesvirus (KSHV; also known as human herpesvirus 8 (HHV8))	Double-stranded DNA herpesvirus	Kaposi's sarcoma, primary effusion lymphoma and some multicentric Castleman's disease	1994	33
Merkel cell polyomavirus (MCV)	Double-stranded DNA polyomavirus	Most Merkel cell carcinoma	2008	34

Human cancer viruses
The first human tumour virus, Epstein–Barr virus (EBV; also known as human herpesvirus 4 (HHV4)), was not described until 53 years after Rous's initial experiments. Anthony Epstein, Bert Achong and Yvonne Barr used electron microscopy in 1964 to identify EBV particles in cell lines from African patients with Burkitt's lymphoma[15]. The unusual geographic distribution of Burkitt's lymphoma had suggested a novel environmental cause, such as a viral infection, which was confirmed by these early virological studies. This spurred other electron microscopy-based searches for human cancer viruses that largely turned out to be fruitless, as cancer viruses generally do not replicate to form virions in tumours (discussed below). The discovery of EBV also sparked contention among cancer biologists because of the difficulty in reconciling the near ubiquitous infection of adults with EBV and the fact that EBV-associated cancers are uncommon[16]. Furthermore, although Burkitt's lymphoma tumours from African and New Guinean patients are almost always positive for EBV, sporadic Burkitt's lymphomas in developed countries frequently lack EBV but retain signature *MYC–IgH* or *MYC–IgL* translocations[17]. This would require a rethinking of disease causality, as EBV does not follow the Galilean principles of causality [18], which require that a virus must be both necessary and sufficient to be the cause of cancer (BOX 2).



Six more cancer viruses have been discovered, in addition to EBV (TABLE 1), which are now widely accepted as causes for invasive human tumours. Additional candidates are continuously proposed but their roles in human cancer remain controversial and unclear. This list is small but we can still draw at least one startling conclusion from it: human cancer viruses do not fall into a single viral class. Much of the past century was devoted to the search for simple human cancer retroviruses similar to the Rous sarcoma virus (BOX 1), but the only retroviruses associated with human cancer are the complex retroviruses: human T-lymphotropic virus-I (HTLV-I) and HIV-1 and HIV-2. HIV does not directly cause cancer but it is frequently included as a cancer-causing agent by virtue of its induction of immunodeficiency, which promotes the development of cancers caused by other viruses[2].

A second surprise is that human cancer viruses span the entire range of virology and include complex exogenous retroviruses (such as HTLV-I), positive-stranded RNA viruses (such as hepatitis C virus (HCV)), DNA viruses with retroviral features (such as HBV) and both large double-stranded DNA viruses (such as EBV and Kaposi's sarcoma herpesvirus (KSHV; also known as human herpesvirus 8 (HHV8)) and small double-stranded DNA viruses (such as HPV and Merkel cell polyomavirus (MCV)). There is no obvious molecular rule that either firmly establishes or eliminates an agent as a potential human tumour virus a priori. Also, almost all of the tumour viruses have close relatives that do not cause human cancer.

a

b

Figure 1 | **Historical figures. a** | The chicken tumour that started it all. This photograph from 1909 shows a sarcoma on the external chest wall of a chicken that was used by Francis Peyton Rous to discover the Rous sarcoma retrovirus[8]. **b** | Vilhelm Ellerman (1871–1924), shown on the left, and his assistant, Oluf Bang (1881–1937), shown on the right. These Danish scientists first succeeded in transmitting a leukaemia-inducing avian retrovirus in 1908. These experiments formed a basis for Rous's subsequent experiments showing a viral cause of a solid cancer. Images courtesy of Medical Museion, Copenhagen University, Denmark.

This leads to the conclusion that almost every virus has the potential to cause cancer but only a very small proportion actually do so.

As suggested above, traditional virological techniques have had limited success in identifying human cancer viruses. EBV virions were identified by cell culture and electron microscopy[19] but this was followed by only one other human tumour virus, HTLV-I[20]. HTLV-I was discovered by Bernard Poiesz, Robert Gallo and colleagues in 1980 (and shortly after confirmed by Yorio Hinuma, Isao Miyoshi and collaborators[21]) from cell lines established from a case of the newly described adult T cell leukaemia/lymphoma (ATLL) syndrome that was previously diagnosed as mycosis fungoides[20,22–24]. This virus was sought by searching for reverse transcriptase activity in a survey of T cell lines, which was partly initiated owing to the earlier discovery that a gibbon retrovirus causes T cell leukaemia. By contrast, HBV, discovered shortly after EBV in the mid-1960s and leading to a Nobel Prize for Baruch Blumberg in 1976, has only recently been successfully propagated in culture and was first linked by serology to acute hepatitis rather than to cancer[25,26]. The role of HBV in hepatocellular carcinoma was established more than a decade later by Beasley *et al.*[27] through longitudinal studies of Taiwanese insurance company cohorts.

The remaining four viruses were discovered as genetic elements using molecular biology, rather than virology, techniques. Since the 1840s, sexual activity had been suspected to be a risk factor for cervical cancer, and Harald zur Hausen reasoned that papillomaviruses might contribute to this cancer owing to their role in sexually transmitted genital warts[28]. He and his colleagues cross-hybridized known papillomavirus DNA to cervical cancer DNA, discovering two novel high-risk papillomaviruses genotypes (HPV-16 and HPV-18) in the early 1980s[29,30] that were subsequently confirmed to be present in most cervical cancers. Similar to HBV, HPV also propagates poorly in culture in most cell types although it is maintained as an integrated, non-productive virus in HeLa cells[29,30]. In the late 1980s, Qui-Lim Choo, Michael Houghton, Daniel Bradley and colleagues sought additional causes for transfusion-transmitted hepatitis (known as the nonA-nonB hepatitis virus) by antibody panning of a randomly primed cDNA library made from the sera of experimentally infected chimpanzees. This led, in 1989, to the isolation of genome fragments from the flavivirus HCV[31], which was then immediately exploited by multiple groups to show that this new virus, like HBV, is associated with hepatocellular carcinoma. HCV was only recently propagated in cell lines[32].

We specifically sought out viral causes for Kaposi's sarcoma and Merkel cell carcinoma (MCC), discovering KSHV in 1994 (REF. 33) and MCV (together with Huicheng Feng from our group) in 2008 (REF. 34). These discoveries were both based on nucleic acid subtraction, although the actual experimental approaches were very different. KSHV was identified by physical DNA subtraction (representational difference analysis)[35] using Kaposi's sarcoma and healthy tissue genomes from the same patient. By contrast, MCV sequences were identified by computational subtraction of cDNA sequence data using digital transcriptome subtraction (DTS)[36], a technique developed by us over a 10-year period and independently developed by others[37]. Applying DTS to MCC revealed viral sequences belonging to a new human polyomavirus. Like most other human cancer viruses, MCV and KSHV are not typically transmissible from the cancers that they help to induce.

These examples show how traditional approaches that are used by virologists, such as virus culture and electron microscopy, often fail in tumour virology. Another difficulty to understanding viruses in human cancer has been the slow realization that

virus infection alone is never sufficient for tumorigenesis, an unsurprising fact that is also true for non-neoplastic viral diseases. Only in a few specific cases, such as KSHV in Kaposi's sarcoma and HPV in cervical cancer, can particular viruses be assumed to be necessary, as they are universally present in these tumours. HBV, HCV and chemical carcinogens each contribute to the total attributable liver cancer risk, but none of these factors alone is required for liver cancer[38]. Thus, the concept of a virus being both necessary and sufficient as the cause of a cancer is too simplistic to be useful in modern cancer research. As cancer is a complex multistep process, it is now obvious that many molecular events, including virus infection, function together to generate the transformed cellular phenotype[39–41].

Immunity is an external factor that has particular importance in determining whether a cancer occurs after exposure to a potential tumour virus. This can be seen with signalling lymphocytic activation molecule-associated protein (SAP) mutations in males that cause immunodeficiency and X-linked lymphoproliferative syndrome after EBV infection[42]. Kaposi's sarcoma, first described in 1872 (REF. 43), also illustrates the importance of immunity to control the expression of a viral cancer. KSHV co-speciated with humans 80 million years ago[44] but infects only ~3% of healthy North Americans[45–50]. Before the AIDS epidemic, KSHV caused less than three Kaposi's sarcoma cases per year per million people in the United States, but rates of Kaposi's sarcoma soared tens of thousands-fold among people with AIDS after the emergence of immune suppression owing to the HIV pandemic[51].

Box 1 | **The long, strange trip of retroviruses in cancer**

"One can scarcely suppose that a horde of viruses, each with its more or less limited potentialities, are passed along together in the ovum or sperm, as generation succeeds generation, or that they reach the young organism by way of the uterus or milk." Francis Peyton Rous, 1960 (REF. 171)

Tumorigenic retroviruses have been central to cancer biology, leading to the development of focus formation assays[172], discovery of reverse transcription[173,174], identification of more than 20 cellular oncogenes[175–177], and ultimately Nobel Prize recognition for Rous 57 years after his initial experiments. The Ellerman and Bang erythroblastosis virus and the Rous sarcoma virus are independently derived from avian leukosis virus (ALV), a simple retrovirus[11,178]. Simple retroviruses can become carcinogenic by recombination with cell-derived oncogenes (*SRC* in the case of Rous sarcoma virus and probably *ERBB2* in the case of the Ellerman and Bang virus), which disrupt the viral genome, usually rendering it non-infectious or by insertional mutagenesis. ALV is endemic among chickens, but Rous sarcoma virus is not and most strains require co-infection with a helper retrovirus to be transmitted[11]. Discovery of endogenous retroviral sequences by Robin Weiss and colleagues[179] suggested that simple retroviruses (endogenous retroviruses (ERV)) could re-emerge from the host genome, raising a perplexing possibility that some oncogenic retroviruses arose during serial cancer transplantation experiments with the unwitting aid of early experimentalists[180,181]. Ironically, tumour viruses helped to identify cellular oncogenes[177] and tumour suppressor genes[182,183], giving rise to the successful somatic mutation theory of cancer that no longer required the confusing biology of tumour viruses. By the 1970s, as Anders Valhne recalls, "…the notion of human cancer viruses became in ill repute and rather than talking of 'human tumour viruses' people in science talked of 'human rumor viruses'." (REF. 24) Discovery of a complex cancer-causing human retrovirus (human T-lymphotropic virus-I (HTLV-I)) in 1980 (REFS 20,24) came too late to fully rehabilitate their reputation.

But the possibility for simple retroviral involvement in human cancer persists. The exogenous ovine Jaagsietke retrovirus has evolved over the past several hundred years from an ERV to cause a transmissible cancer of in-bred sheep — most famously in the cloned sheep Dolly[184]. Spontaneously arising human ERV-derived retroviruses might similarly contribute to sporadic human cancers but would be difficult to detect and non-transmissible[185]. Further, a recent candidate human cancer virus is a simple retrovirus that is controversially associated with prostate cancer and chronic fatigue syndrome[186–188]. Time, careful epidemiology and careful experimentation will determine the role of these viruses in human cancer.

Indirect versus direct carcinogenesis

Infectious cancer agents (including, viruses, bacteria and parasites) have been divided into two broad categories: direct carcinogens, which express viral oncogenes that directly contribute to cancer cell transformation, and indirect carcinogens that presumably cause cancer through chronic infection and inflammation, which eventually leads to carcinogenic mutations in host cells[52,53]. This is a useful description for infectious cancer causes that will undoubtedly change as knowledge accumulates. By definition, a direct viral carcinogen is present in each cancer cell and expresses at least one transcript to maintain the transformed tumour cell phenotype, as occurs with HPV-, MCV-, EBV- and KSHV-related cancers. Evidence supporting this comes from knockdown studies in which the loss of viral proteins results in the loss of host cancer viability[54–60]. Indirect carcinogens (most notably, the *Helicobacter pylori* bacterium) could potentially also include 'hit-and-run' viruses in which the viral genes are lost as the tumour begins to mature, although good examples of this process have not been documented to date.

Several agents (such as HBV, HCV and HTLV-I), however, do not fit neatly into either the indirect or the direct carcinogen categories. Hepatocellular carcinoma (HCC) generally arises after prolonged liver cirrhosis from chronic virus-induced cell death and regeneration[61–63]. HBV is clonally integrated into the genomes of tumour cells in almost all HBV-related cancers, but it is not clear whether persistent HBV (or HCV) gene expression is required for HCC cell proliferation[61]. HTLV-I, like most direct carcinogens, is present as a clonal infection of ATLL, but expression of its putative oncogene *v-tax* is frequently absent in the mature leukaemia or lymphoma cells[64]. Transgenic models reveal that various proteins from these viruses, including the HBX protein from HBV, NS5 protein from HCV and TAX from HTLV-I can initiate oncogenic transformation[62,65]. Thus, for these viruses it remains unclear whether specific viral products maintain mature tumour cells, promote a precancerous cell phenotype or contribute to cancer solely through prolonged infection and chronic inflammation[62,65]. Bacterial carcinogens can also have features that are reminiscent of direct carcinogens[66], showing that the simple dichotomization of direct and indirect carcinogens is probably inadequate.

The indirect versus direct paradigm is nonetheless very useful as it guides our thinking about which cancers are most likely to harbour a new human cancer virus. Cancers that are related to immunosuppression are candidates for being caused by tumour viruses[67]. Loss of surveillance for specific viral cytotoxic T cell epitopes without generalized immunosuppression, as might occur during ageing, is also likely to promote cancers that are caused by viruses[68,69]. This makes intuitive sense, particularly for direct carcinogens, as they must express at least one foreign protein in each cancer cell, but even cancers caused by indirect infectious carcinogens have an increased occurrence in immunosuppressed populations[67]. In a classic epidemiological study, Beral *et al.*[70] used this knowledge of direct and indirect cancer causation on registry

Box 2 | **The EBV–cancer paradox**

Resolving the Epstein–Barr virus (EBV)–cancer paradox — that a common infection can cause a rare cancer — required the recognition that chronic viral infection functions together with multifactorial non-viral risks to contribute to cancer. The well-known Koch's postulates are not applicable to viruses such as EBV, which generally cannot be isolated as pure cultures *in vitro* or used to re-infect susceptible laboratory animals[189,190]. Hill's criteria[191], used to determine the relationship between cigarette smoking and lung cancer, work well for uncommon agents, such as Kaposi's sarcoma herpesvirus (KSHV) that causes Kaposi's sarcoma[108], but also have unstated pathobiological biases that limit their use in tumour virology. Subsequent to its discovery, Werner and Gertrude Henle and colleagues showed that EBV infection can immortalize primary B cells[192], a property that is unique to EBV; its oncogenes were characterized[193]; tumour-viral clonality was established[94]; and additional examples of EBV-associated cancers and lymphoproliferative diseases were described[194]. From the perspective of Bayesian reasoning, the posterior probability that EBV causes cancer was strengthened by the accumulating clinical and basic data from various sources that ultimately left little doubt that EBV has a causal role in specific tumours. Nonetheless, more than 30 years passed from its discovery until it was officially declared a human carcinogen by an international cancer agency[195].

data of patients with AIDS to correctly predict most of the major epidemiological features for the virus (KSHV) that caused Kaposi's sarcoma before its actual discovery. Similarly, analyses by Engels *et al.*[71] of data of patients with AIDS focused our attention on MCC as possessing a potentially infectious origin. Other immunosuppression-related tumours, including non-melanotic skin tumours, EBV-negative Hodgkin's disease and non-Hodgkin's lymphomas, are promising candidates for future cancer virus discovery[72,73].

This paradigm not only suggests where to look for human cancer viruses but also how to look for them. A cell possesses approximately 200,000 mRNA transcripts, and methods to sequence cDNA substantially beyond this level are readily available. If a direct carcinogen is present and expresses a foreign oncoprotein, carrying out DTS on cDNA from a monomorphous tumour specimen is likely to sequence a viral gene. There are technical difficulties with this approach (particularly in recognizing that a transcript belongs to a novel virus rather than being a missequenced or unannotated human transcript, as outlined elsewhere[36]) that place constraints on sequencing-based virus discovery. As genomic databases improve, molecular distinctions between self and non-self genomes will become more precise and easier to detect. Equally importantly, sequencing technologies can help to exclude a direct carcinogen if it is not present in a cancer. Deep sequencing of four human mesothelioma tumour cDNAs failed to identify SV40 viral transcripts[74], providing evidence against a long-standing hypothesis that SV40, a rhesus polyomavirus, is directly involved in the development of mesothelioma[75]. Although this does not exclude SV40 as a cause of human cancer, this virus would have to do so in mesotheliomas through a new and undescribed mechanism. Tumour trasnscriptome sequencing can also be paired with sequencing of the appropriate control tissues to determine cancer cell gene expression. Therefore, if properly carried out, even negative searches for viral sequences can provide useful clues about the origins of human cancer.

Latency and pseudo-latency

A common feature for human tumour viruses is that they are persistent latent or pseudo-latent infections that generally do not replicate to form infectious virus particles in tumours. All of the viruses in TABLE 1 have the capacity to form virions and become transmissible at some point in their natural lifecycles, but within tumours these infections are generally latent so that productive virus replication (also known as lytic replication) is either diminished or absent[76]. Viral latency serves as an immune evasion strategy allowing the virus to hide from the immune system by turning off unnecessary viral proteins that might be sensed by cell-mediated immune recognition. The virus persists as a naked nucleic acid, often as a plasmid or episome, which relies on host cell machinery to replicate whenever the cell divides. Viral latency should not be confused with clinical latency, which means asymptomatic infection. Latent viral infections can be symptomatic, as in viral cancers, and active lytic viral replication can be relatively asymptomatic, as occurs during the prodromal phases of HIV or HCV infection. As early as the 1970s, investigators recognized an inverse relationship between virus replication, or permissiveness, and cell transformation for tumour viruses. SV40, for example, transforms human cells efficiently only when mutations are introduced into its replication origin to prevent viral replication[77]. The discovery of EBV in Burkitt's lymphoma was fortuitous as most of the tumour cells harbour EBV DNA in a non-transmissible episomal form. Rare herpesvirus-like structures were also seen by electron microscopy in Kaposi's sarcoma tumours as early as 1984 (REF. 78) but the vast majority of KSHV-related tumours silently harbour KSHV as latent genomes[79–81].

The most likely explanation for the connection between virus latency and tumorigenesis is that productively replicating viruses initiate cell death, which has long been known to virologists as the cytopathic effect (CPE). Counter-intuitively, from the point of view of tumour virology, virus-induced CPE can be harnessed to kill cancer cells in viral oncolytic therapies, illustrating the anticancer activity of active lytic viral replication[82,83]. Although CPE is frequently thought of as a virus-induced event, it is actually a stereotypical and nonspecific innate immune response of cells to infection by many types of viruses. When latent viruses switch to producing virions, virus replication generates pathogen-associated molecular patterns from partially synthesized viral chromosomes, double-stranded RNAs and empty capsids that trigger cellular DNA damage responses and innate immune signalling[84–86]. For some viruses, lytic replication generates a linear viral chromosome that can be recognized as a DNA fragment[87] unless either the DNA ends are structurally hidden from DNA damage response sensors by encapsidation or these sensors are inactivated. Activation of toll-like receptor and interferon signalling by virus infection initiates and amplifies this innate immune response[88]. Together, these cellular responses generally kill infected cells that are undergoing productive virus replication — hence the term lytic replication. Once triggered, lytic viral replication is largely irreversible and initiates a race between the virus to successfully reproduce itself and the death of the host cell.

Among viruses, latency is best understood for the herpesvirus family (particularly for EBV and KSHV that have latent viral tissue culture systems), in which it is tightly regulated by transcriptional repression[89]. Latent herpesviral protein expression is limited to a few crucial, non-structural viral products that include oncogenic proteins and microRNAs (miRNAs). During herpesviral latency the viral genome is not packaged into virions but instead the viral genome replicates in tandem with the host cell using the replication machinery of the cell and is tethered to chromosomes as a naked circular genome[90].

Lytic replication to produce infectious virions is initiated through a highly stereotypical series of viral transactivator cascades, which are cued by cellular environmental signalling pathways, that leads to host cell death and the release of infectious virions[91]. Although not fully described, these cellular environmental triggers might tell the persistent virus when to initiate lytic virus replication to optimally achieve transmission to a new host and survive.

Viral control of lytic and latent replication is less well-understood for the RNA and small DNA tumour viruses, but these agents also show a similar absence of productive viral replication during malignancy. HTLV-I is maintained as an integrated DNA provirus that is largely transcriptionally silent within ATLL cells[22,65]. In these tumours, the viral oncoprotein TAX is thought to promote early precancerous cell expansion and survival, but it may not be required in the fully malignant T cell. However, another candidate HTLV-I oncoprotein, HBZ, continues to be expressed in mature ATLL cells and might have a role in maintaining cell transformation[92]. Among the small DNA tumour viruses, fragmentation and integration of viral DNA into the nascent tumour cell eliminates their ability to replicate as virions, a state that we have termed 'pseudo-latency'. Integration provides primary evidence for cancer causation if the integrated virus is clonal within individual tumours, as occurs in some of the HPV-, MCV-, HBV- and HTLV-I-related malignancies[30,34,61,93]. Although herpesviruses do not generally integrate into the host genome, recombination patterns for their terminal repeat sequences can also be used as markers for tumour cell clonality[94,95]. In such a scenario, clonality is a piece of molecular evidence placing the suspect virus at the 'scene of the crime'.

As viruses in tumours are generally latent, antiviral drugs targeting the viral replication machinery are ineffective in treating mature tumours. However, antiviral therapy can in some instances prevent the development of new tumours. Randomized clinical trial data show, for example, that targeting the KSHV thymidine kinase and phosphotransferase proteins[96] can prevent >90% of new Kaposi's sarcomas from forming[97] but this targeting has no effect on established tumours[98]. A possible exception to the rule for viruses being non-replicative and silent in human malignancy is HBV. This virus can infect nearly all hepatocytes in the liver during acute HBV hepatitis but most cells survive infection, eventually clearing the viral genomes without cell death[99]. Intriguingly, non-cancerous liver tissues from patients with HBV-associated HCC show patterns of microclonality[63]. It is not known whether this results from re-expanding clones of hepatocytes or whether it represents a premalignant change.

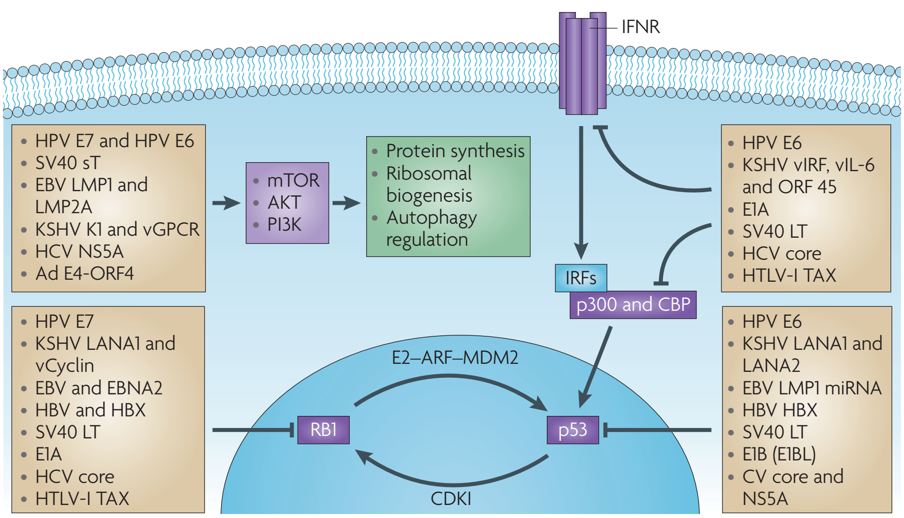


Figure 2 | **Common cellular targets for unrelated tumour virus oncoproteins.** An incomplete but diverse list of animal and human tumour virus proteins that target RB1, p53, interferon and PI3K–mTOR signalling pathways. Most of these viral proteins are evolutionarily distinct from each other and have unique mechanisms for regulating or ablating these signalling pathways. Convergent evolution of tumour viruses to target these (and other cellular signalling pathways (not shown), including interleukin-6 (IL-6)–signal transducer and activator of transcription 3 signalling, telomerase and nuclear factor-κB (NF-κB) signalling pathways) reveals commonalities among the cancer viruses in tumour supressor and oncoprotein targeting. CBP, cAMP-response element binding protein; CDKI, cyclin-dependent kinase inhibitor; EBV, Epstein–Barr virus; HCV, hepatitis C virus; HPV, human papillomavirus; HTLV, human T-lymphotropic virus; IFNR, interferon receptor; IRF, interferon regulatory factor; KSHV, Kaposi's sarcoma herpesvirus; LMP, latent membrane protein; miRNA, microRNA.

Origins of viral oncogenes

Cancers caused by viruses — such as non-infectious cancers — are biological accidents. Tumours do not increase transmissibility of viruses or enhance their replication fitness. A common misperception is that cancer viruses cause cancer to increase viral burden and transmission. Instead, tumours are 'dead-end' events for viruses. Only a small proportion of people infected with any of the human tumour viruses develop tumours and, of those people who do, they rarely (if ever) serve as sources for ongoing transmission. Instead, most human tumour virus transmissions are asymptomatic or mildly symptomatic but do not lead to neoplasia.

If we discard the idea that viruses are evolutionarily programmed to cause cancer, then why do tumour viruses encode oncogenes? There is strong selection to maintain viral genes that can initiate tumorigenesis, as diverse viruses (including, non-tumour viruses) show remarkable convergence to target the same tumour suppressor pathways (FIG. 2). For example, most of the human tumour viruses encode oncoproteins that target RB1 and p53, although they do so through different and unique mechanisms[100]. Other common targets that have roles in tumorigenesis for tumour viruses include telomerase reverse transcriptase (TERT[101–105]), cytoplasmic PI3K–AKT–mTOR[106], nuclear factor-κB (NF-κB)[59,64,107–109], β-catenin (also known as CTNNB1)[110] and interferon signalling pathways[111].

Two widely held views exist for the teleology of viral oncogenes (FIG. 3). The first hypothesis originated from the biology of small DNA tumour viruses (such as HPV and SV40) and was based on the presumed need for these viruses to re-initiate the cell cycle entry of differentiated cells to set conditions for viral replication[100,112]. Because the host replication machinery and nucleotide pools are limited in the G0 cell cycle phase of differentiated cells, these viruses might force unscheduled S phase entry to generate the cellular resources that are needed for viral genome replication. Disruption of cell cycle regulation, however, also activates cell death signalling pathways, such as p53, and so apoptotic signalling should also be inhibited to allow the efficient manufacture and export of viruses before cell death.

This is illustrated by the lifecycle of HPV, which infects basal epithelial cells that differentiate into arrested squamous epithelium.

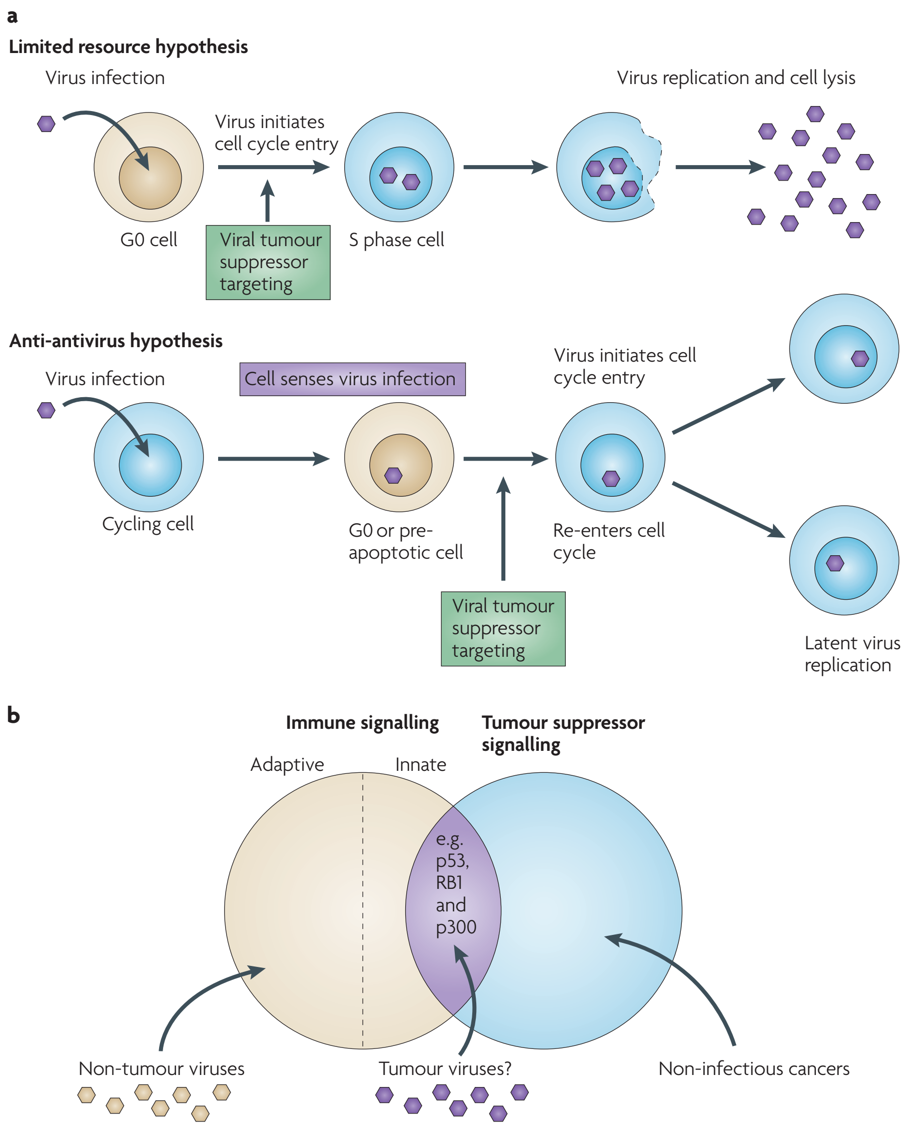


Figure 3 | **Two views for the origins of viral oncoproteins. a** | The tumour virus proteins target RB1 and p53 to drive a quiescent G0 cell into S phase of the cell cycle, allowing viral access to the nucleotide pools and replication machinery that are needed for replication and transmission[100]. Viral tumourigenesis is a by-product of the molecular parasitism by viruses to promote their own replication. Cells respond to virus infection by activating RB1 and p53 to inhibit virus replication as part of the innate immune response[86]. To survive, tumour viruses have evolved the means for inactivating these and other immune signalling pathways that place the cell at risk for cancerous transformation. This view holds that many tumour suppressor proteins have dual functions in preventing cancer formation and virus infection. **b** | An illustration of the overlap between intracellular innate immune and tumour suppressor signalling. Under typical circumstances, viruses do not cause cancers except in the settings of immunosuppression and/or complementing host cell mutations. Non-tumorigenic viruses, which constitute the overwhelming majority of viruses, target many of the same innate immune and tumour suppressor pathways as tumour viruses but do so in ways that do not place the host at risk for carcinogenesis. Apart from p53, RB1 and p300, additional proteins are likely to have both tumour suppressor and innate immune functions.

A commonly held view of HPV targeting of tumour suppressor pathways is that as the infected keratinocyte differentiates, the HPV E7 oncoprotein inactivates RB1 signalling to drive quiescent, infected cells back into a proliferative state, thus allowing viral genome replication[113]. Simultaneously, the HPV E6 protein induces ubiquitin-mediated degradation of p53, preventing the premature apoptosis that would otherwise limit the efficiency of virus production[114]. Under normal circumstances, these virus-deregulated cells will typically be sloughed off together with infectious virions. Rare mutations, however, that disrupt this lifecycle (such as an HPV integration event that results in the loss of early viral gene regulation) can set the stage for this molecular parasitism to turn into cancer cell transformation. By targeting the cell cycle checkpoints and anti-apoptotic machinery that are involved in genomic proofreading, viral oncogenes also induce cellular genomic instability and aneuploidy, which in turn contribute to carcinogenesis[115,116]. In summary, the most commonly held view for the function of viral oncogenes is that these genes target cellular tumour suppressor pathways to promote productive viral replication and only contribute to cancer when random mutations disrupt this equilibrium.

However, studies on the large DNA tumour viruses (such as EBV and KSHV) suggest a more complex interaction between the viral oncogenes and the host cell that may have more to do with evading immune responses during latency than ensuring viral genome replication[86,111,117]. During lytic viral replication, these viruses also hijack the cell cycle regulation machinery to promote their own genomic replication. The oncogenic herpesviruses encode proteins to inhibit p53, RB1 and other tumour suppressor checkpoints during active lytic viral replication[118–121], and also possess virally encoded DNA synthesis enzymes[95]. These viral genes, like their counterparts among the small DNA tumour viruses, set the stage for the rapid replication and amplification of viral genomes by generating an S phase-like cellular state that can replicate viral DNA once lytic replication is initiated.

But the viral proteins and virus-encoded miRNAs that drive herpesviral tumours are expressed during latency, and these viral oncogenes can not directly contribute to productive viral replication[108,122–126]. Herpesvirus oncoproteins are expressed at the wrong time for them to be involved in generating the cellular resources needed for virus genome replication. The KSHV LANA1 oncoprotein suppresses lytic replication to maintain virus latency while it simultaneously targets RB1, p53 and interferon signalling responses[127–129]. The KSHV-encoded cyclin is another latent viral oncoprotein[130,131] that is expressed in a cell cycle-dependent manner[132]. Similar to HPV E7, it targets RB1 and inactivates the G1/S checkpoint but it does not promote virus replication. For EBV, at least three classes of viral latency have been established, which are distinguished by different groups of oncogenic non-coding RNAs, Epstein–Barr virus nuclear antigens (EBNAs) and latent

membrane proteins (LMPs)[133]. These EBV latent products target cell cycle and apoptotic signalling pathways (for example, p53-upregulated mediator of apoptosis (PUMA) is targeted by a latent EBV miRNA[125]), but are also not directly involved in generating EB virions or amplifying EBV genomes during productive virus replication. Studies from the herpesviruses raise the question: what are the viral oncoproteins doing if they are not preparing the cell for virus replication?

Viral oncogenes and immune evasion

Over the past decade, increasing evidence has indicated that the evasion of innate immunity also plays a fundamental part in viral tumorigenesis. Humans, as well as most complex metazoans, are chimeric for numerous viruses. In some cases, mammals may even exploit latent viral infections to beneficially regulate their own innate immune systems[134]. So, it is not surprising that major portions of the eukaryote cell are devoted to protecting the host genome from foreign viral sequences. Innate immune signalling shares many similarities to tumour suppressor signalling, as both processes initiate cell cycle arrest and prime apoptotic pathways. Key effector proteins such as the p21 cyclin-dependent kinase inhibitor[135] and p53 (REF. 136) are shared by both tumour suppressor and innate immune surveillance signalling networks. This suggests that targeting of tumour suppressor pathways by viruses may actually represent an immune evasion response that disables antiviral pathways but inadvertently places the infected cell at risk for cancerous transformation (known as the anti-antivirus hypothesis)[86,111].

The dual nature of innate immune signalling in antivirus and anticancer functions is illustrated by interferon regulatory factors (IRFs), a family of induced and immediate-early transcription factors that regulate interferon transcriptional responses[137–139]. KSHV encodes four IRF homologues[95,140], including vIRF1 (REF. 141), which behaves similarly to IRF2 (REF. 139) by inhibiting interferon signalling and initiating cell transformation. Most of the other established KSHV oncoproteins, including interleukin-6 (vIL-6; also known as K2)[142], FLICE inhibitory protein (vFLIP; also known as ORF71)[109], ORF K1 protein[143], latent nuclear antigen 1 (LANA1)[129] and LANA2 (REFS 144,145), also have well defined innate immunomodulatory roles[86,117]. For EBV, RNA-dependent protein kinase (PKR) immune signalling is targeted by latent small non-coding EBV RNAs (EBERs)[146] that might have a role in EBV-induced tumorigenesis. This implies that infected cells can sense latent virus infection and must deactivate cell cycle arrest and pro-apoptotic pathways to survive in the hostile environment of the cell.

Although a role for oncoproteins in innate immune evasion is best characterized for herpesviruses, other viral oncoproteins, such as the human adenovirus E1A oncoprotein that causes cancer in rodents, also dually inhibit interferon signalling and tumour suppressor pathways by targeting the histone acetyltransferases p300 and CBP[147,148], which participate in interferon-induced transcription. The relationship between tumour suppression and cellular antiviral activity was described by Takaoka and colleagues who showed that knock out of *Trp53* (encoding p53) causes immune deficiency to virus infection, and that virus-induced inflammatory cytokines prime cellular pro-apoptotic signalling pathways[136]. p53 seems to not only be the 'guardian of the genome' (REF. 149) but also a guardian against viral infection.

Other cellular pathways (FIG. 3a) with roles traditionally ascribed to preventing tumour cell formation might also play a part in immunity to viral infection[150]. Cellular sensors for DNA and RNA ends are generally studied as triggers for the repair of somatic mutations but they also have a role in sensing viral nucleic acids[151,152]. DNA damage responses are activated during viral uncoating and replication[65,153–155] that can lead to cell cycle arrest and inflammatory signalling activation[156]. Disarming the antiviral targeting of tumour suppressor signalling may allow the prolonged persistence of viral infection but also carries obvious risks for generating a cancer. Despite evidence for a relationship between innate immune and tumour suppressor signalling (FIG. 3b), the mechanisms that cells might use to sense episomal viral genomes, as occurs during latent herpesvirus infection, remain unknown.

The newest member of the club: MCV

The examples we describe above mainly come from the first six human tumour viruses. How does the most recently discovered virus, MCV, compare? Similar to SV40 and murine polyomaviruses, MCV encodes a multiply spliced tumour (T) antigen protein complex that targets several tumour suppressor proteins[157]. As with EBV and Burkitt's lymphoma, MCV is present in most MCCs and it is a near-ubiquitous infection of adults[158–162]. Within infected tumours, the T antigen is only expressed in tumour cells, as predicted for a direct viral carcinogen[163]. Given these features, the strongest evidence to support MCV causing MCC comes from its random clonal integration into Merkel cell tumours[34,164] and knockdown studies showing that T antigen expression is required for the survival of virus-positive

Glossary

Antibody panning
cDNA from a tumour is used to express proteins in bacteria and transferred to replicate filters. Antibody screening of the filters can then be used to identify colonies expressing the specific cDNA encoding an antigen.

Bayesian reasoning
A scientific approach developed from Bayes theorem, combining features of the Logical Positivist and Kuhnian schools of science philosophy, and describing how the probability of a hypothesis (in this case, virus A causes cancer B) changes with new evidence. In simple terms, it can be described as the repeated application of the scientific method to falsify a hypothesis such that the hypothesis has a high probability of being either true or false.

Digital transcriptome subtraction
DTS. Method to discover new viruses by exhaustively sequencing cDNA libraries and aligning known human sequences by computer leaving a smaller candidate pool of potential viral sequences for analysis[36].

Endogenous retrovirus
ERV. Retrovirus that has inserted into the metazoan germline genome over evolutionary timescales and is now transmitted to offspring as a genetic element through Mendelian inheritance. Approximately 8% of the human genome is estimated to be derived from retroviral precursors.

High-risk papillomaviruses
More than 160 different genotypes or strains of HPV have been described but only a few genotypes belonging to a high-risk carcinogenic clade of the α-HPV genus are responsible for invasive HPV-related anogenital cancers[211].

Longitudinal study
Virus infection is measured initially in a cohort of patients who are then followed over time to determine cancer occurrence.

Prodromal phase
An early set of nonspecific symptoms that occur before the onset of specific disease symptoms.

Representational difference analysis
A PCR-based subtractive hybridization technique that can subtract common human sequences from a tumour genomic library using a control human tissue genomic library[35].

Serology
The measurement of antibodies against viruses in blood or bodily fluids. This usually does not distinguish ongoing infections from past viral infections.

Merkel cell lines[58]. Although skin carriage of the virus is common[165], no other tumours except MCC have yet been convincingly linked to MCV infection. As SV40 and related polyomaviruses have been workhorses for cancer research from the early 1960s, studies on these viruses can be directly applied to MCV, allowing rapid progress in understanding its biology in humans.

MCV is intriguing because the precise molecular events leading to cancer have been described and they help to explain why this common childhood infection can lead to a rare cancer that is associated with sun exposure (FIG. 4). The initial event in MCV-driven MCC carcinogenesis is likely to be the loss of immune surveillance for the virus. MCC principally occurs in the elderly and immunocompromised, and those people developing MCV-related MCC have greatly increased antibodies to MCV structural proteins, suggesting that the loss of cellular immune control over the virus may allow viraemia before the development of the tumour[159,161,162].

The virus then undergoes at least two mutations, the first being non-homologous recombination with the host chromosome. As with other tumour viruses, clonality of integration in primary tumours and their metastases shows that this occurs before the tumour cell begins to proliferate[34]. As MCV has no mechanism to excise its genome, the virus cannot replicate and is no longer transmissible, and this is analogous to HPV in most cervical carcinomas.

MCV integration, however, generates a problem for the nascent tumour cell. The viral large T antigen not only targets tumour suppressor molecules, such as RB1, but it is also required for productive virus replication. During a typical infection by MCV, the large T antigen protein binds to the viral genome and its helicase domain unwinds the viral origin to allow DNA replication[166]. If the full-length large T antigen protein is expressed in tumour cells with an integrated virus, it initiates unlicensed DNA synthesis at the integration site causing replication fork collisions and DNA break responses that could lead to cytopathic cell death[157]. All MCV genomes that have been obtained from tumours so far, however, have inactivating secondary mutations in the T antigen gene that eliminate its DNA replication capacity. Whether additional cellular mutations are required for the successful outgrowth of MCV-infected MCC tumours is currently unknown.

Molecular evolution of MCV in MCC tumours illustrates many of the common features that have been described for the other human tumour viruses. Most notably, tumour formation is a rare, accidental occurrence in the lifecycle of this otherwise innocuous virus. The recent discovery of additional new human polyomaviruses[165,167,168] provides the opportunity to determine whether other members of this group share a similar potential for contributing to human viral cancers.

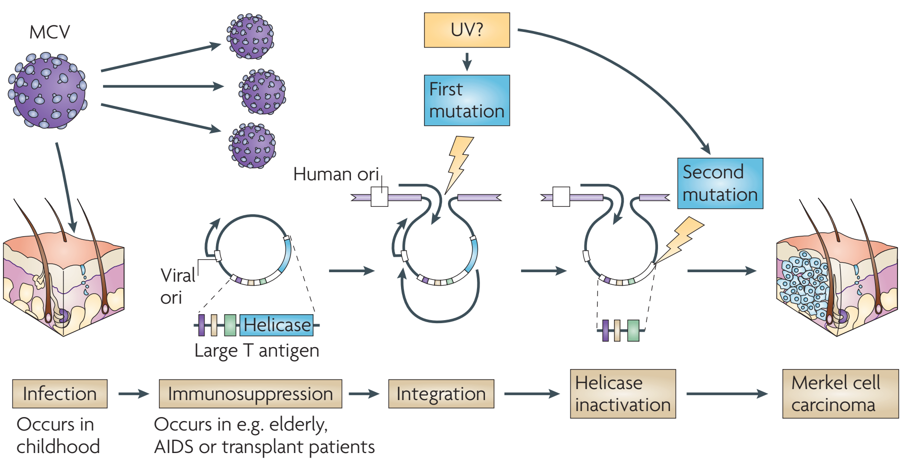


Figure 4 | **The molecular evolution of a human tumour virus.** Merkel cell polyomavirus (MCV), which has tumour-specific truncation mutations, illustrates common features among the human tumour viruses involving immunity, virus replication and tumour suppressor targeting. Although MCV is a common infection, loss of immune surveillance through ageing, AIDS or transplantation and subsequent treatment with immunosuppressive drugs may lead to resurgent MCV replication in skin cells[161]. If a rare integration mutation into the host cell genome occurs[34], the MCV T antigen can activate independent DNA replication from the integrated viral origin that will cause DNA strand breaks in the proto-tumour cell[157]. A second mutation that truncates the T antigen, eliminating its viral replication functions but sparing its RB1 tumour suppressor targeting domains, is required for the survival of the nascent Merkel tumour cell. Exposure to sunlight (possibly ultraviolet (UV) irradiation) and other environmental mutagens may enhance the sequential mutation events that turn this asymptomatic viral infection into a cancer virus.

Future directions

The reduced cost and increased accuracy of sequencing technologies has created the opportunity for most research groups to search for cancer viruses. Only a snippet of unique nucleic acid sequence is needed to discover a new human tumour virus and to begin characterizing it, so the pool of cancer-causing candidates is almost certain to grow in the coming decade. Equally importantly, the reliability of human sequence databases has matured to a level at which certain classes of cancer agents might be excluded when none is found. Identifying a new virus, however, is only the beginning in determining whether it causes human cancer. Cancer causation theories work well for uncommon viruses that are uniformly present in a particular type of cancer[64,108]. Head and neck squamous cell carcinomas[169] and MCCs[34] are examples of cancers that were previously assumed to be homogenous cancers but are now recognized as likely to be caused by both infectious and non-infectious (or at least not identified infectious) aetiologies. Epidemiologists will be increasingly pressed to determine whether a candidate viral agent might cause only a small but important portion of a type of tumour. New epidemiological methods that make better use of molecular biologic data will be key to resolving the causes for these cancers.

Viruses have had a chequered history in cancer biology over the past century. Depending on the time and the fashion, viruses have been either sought out as the primary cause for cancer, or ignored as inconsequential to this disease. We are now entering a more mature phase of research with the realization that a considerable proportion of cancers are indeed caused by viruses. For these cancers, infection is only one component in their ultimate cause. But failure to recognize the importance of viral cancers has led to overlooked opportunities in cancer control. Despite EBV being the first discovered human tumour virus, there is no EBV vaccine and little enthusiasm for its development. KSHV has emerged as a leading cause of adult cancer in sub-Saharan Africa[170] but no movement has yet been made in developing clinical interventions against this virus or its cancers.

The development of anti-latent viral drugs and immunological therapies against cancer virus antigens are achievable goals that have not yet been pursued in modern cancer control. The real measure of success for the past century of tumour virus research will be the future exploitation of existing research to effectively diagnose, treat and prevent cancers that are caused by viruses.

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doi:10.1038/nrc2961

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Acknowledgements
The authors would like to thank G. Klein and O. Gjoerup for helpful comments and corrections to the manuscript; M. Melbye at the Danish Statens Serum Institut, and T. Söderqvist and his staff at the Medical Museion, University of Copenhagen, Denmark, for materials; and F. Zappa for help in preparing the manuscript. The authors are supported by NIH CA136363, CA120726, the Al Copeland Foundation, and American Cancer Society Research Professorships.

Competing interests statement
The authors declare competing financial interests; see web version for details.

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Human papillomavirus oncoproteins: pathways to transformation

Cary A. Moody and Laimonis A. Laimins

Abstract | An association between human papillomavirus (HPV) infection and the development of cervical cancer was initially reported over 30 years ago, and today there is overwhelming evidence that certain subtypes of HPV are the causative agents of these malignancies. The p53 and retinoblastoma proteins are well-characterized targets of the HPV E6 and E7 oncoproteins, but recent studies have shown that the alteration of additional pathways are equally important for transformation. These additional factors are crucial regulators of cell cycle progression, telomere maintenance, apoptosis and chromosomal stability. Understanding how HPV oncoproteins modify these activities provides novel insights into the basic mechanisms of oncogenesis.

Papanicolau smear
A method used to screen for the cellular changes that accompany HPV infection and used as a diagnostic for HPV-induced disease.

Episome
An extrachromosomal DNA element such as a plasmid that can replicate independently from host chromosomal DNA.

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doi:10.1038/nrc2886
Published online 1 July 2010

Human papillomaviruses (HPVs) are small DNA viruses that show a tropism for squamous epithelium. Over 120 types of HPV have been identified and approximately one-third of these infect the squamous epithelia of the genital tract[1]. Of the genital HPVs, which are sexually transmitted, 15 are categorized as high risk and are considered the causative agents of most cervical cancers, with over 99% of cervical lesions containing viral sequences[2]. The remaining viral types are rarely found in malignancies. High-risk HPVs are also associated with many penile, vulvar and anal carcinomas and contribute to over 40% of oral cancers[3]. A vaccine has recently been introduced that can prevent the initial infection by two of these high-risk types, HPV 16 and 18, which are responsible for about 70% of cervical cancers (BOX 1). The HPV types responsible for the remaining 30% of cancers are not yet included in the vaccines. Although Papanicolau smears (PAP smears) have reduced the incidence of cervical cancer by over 80% in the United States, cervical cancer is the second leading cause of cancer deaths in women worldwide, and effective implementation of the HPV vaccine and continued screening should dramatically reduce the incidence of these cancers. The process by which HPV facilitates tumour initiation and fosters tumour progression is an exceptional model to understand the development of many other human cancers and also allows identification of additional signalling pathways targeted in malignant progression. The importance placed on understanding the contribution of HPV infection to malignant progression is highlighted by the 2008 Nobel Prize to Harald zur Hausen for his discovery that high-risk HPV types are the causative agents of cervical cancer.

Given that not everyone infected with high-risk HPVs develops cancer, additional genetic alterations are needed for malignant progression[4]. The HPV oncoproteins E5, E6 and E7 are the primary viral factors responsible for initiation and progression of cervical cancer, and they act largely by overcoming negative growth regulation by host cell proteins and by inducing genomic instability, a hallmark of HPV-associated cancers. This Review summarizes current knowledge of the mechanisms used by these HPV oncoproteins to promote tumorigenesis.

Productive viral life cycle

To understand how HPV infection results in malignant development, it is important to first describe the unusual life cycle of these viruses (FIG. 1). This will help to understand how the tumour-promoting activities of E5, E6, and E7 have evolved from their functions in productive viral replication. Most viruses infect a target cell and produce progeny virus from that same infected cell. Conversely, in HPV infections, the synthesis of new virions occurs only after the infected cell has undergone mitosis and one of the infected daughter cells has differentiated[5]. HPVs infect cells in the basal layer of stratified squamous epithelia. These basal cells become exposed as a result of small microwounds and are the only proliferating cells in normal epithelia, as differentiated cells in the suprabasal layers have exited the cell cycle. Following infection, HPV genomes are established as extrachromosomal elements or episomes. HPV genomes are small (approximately 8 kb in size) and do not encode polymerases or other enzymes necessary for viral replication. HPVs must therefore rely on the host

At a glance

- Human papillomaviruses (HPVs) are the causative agents of over 99% of cervical cancers. Cervical cancer is the second largest cause of cancer deaths in women worldwide.
- Infection by high-risk HPV types is necessary but not sufficient for progression to cancer. Mutations in cellular genes and chromosomal rearrangements induced by genomic instabilities are important contributing events.
- HPV E6 and E7 are the primary transforming viral proteins and E5 enhances proliferation and may contribute to cancer progression.
- A primary target of E7 is the retinoblastoma (Rb) family of proteins that control the activity of E2F transcription factors, which are key regulators of S phase genes. Inactivation of Rb is important for the differentiation-dependent productive viral lifecycle and for tumour progression.
- The efficient abrogation of Rb function by E7 leads to increased levels of p53 and, consequently, the E6 proteins have evolved to target p53 for degradation. E6 also activates telomerase expression and modulates the activities of PDZ domain-containing proteins and tumour necrosis factor receptors.
- E7 proteins also alter cell cycle control through interactions with histone deacetylases, cyclins and cyclin-dependent kinase inhibitors.
- E6 and E7 induce genomic instability through multiple mechanisms, including aberrant centrosome duplication.
- E6 and E7 also target cytokine expression to modulate cell proliferation and interferon responses, contributing to immune evasion.
- E5 binds to B cell receptor-associated protein 31 in the endoplasmic reticulum to control trafficking of proteins and to the vacuolar ATPase in endosomes to modulate epidermal growth factor receptor turnover and maintain constitutive signalling.

cell replication proteins to mediate viral DNA synthesis. In HPV infections, suprabasal cells remain active in the cell cycle as they undergo differentiation, and a subset of cells re-enter S phase in the top epithelial layers to replicate HPV genomes in a process called amplification[6]. This is followed by capsid protein synthesis, virion assembly and release.

The proliferation capacity of these HPV-infected cells is uncoupled from differentiation and is controlled by various cellular factors, the most prominent of which are members of the retinoblastoma (Rb) family, consisting of p105 (RB), p107 and p130. The HPV E7 protein binds to Rb family members and targets them for degradation[7]. This results in the release and activation of E2F transcription factors that drive expression of S phase genes. E7 proteins from all HPV types bind Rb family members but the high-risk E7 proteins do so with much higher affinity[8,9]. The efficient binding of Rb by E7 can lead to inhibited cell growth and apoptosis through a p53-dependent pathway. As a result, high-risk E6 proteins have evolved to target the tumour suppressor p53 for degradation, preventing cell growth inhibition in both undifferentiated and differentiated cells.

The combined action of high-risk E6 and E7 proteins in targeting these cell cycle regulators to maintain S phase competence in differentiating cells also results in abrogation of many cell cycle checkpoints. In cells persistently infected with HPV, this leads to the accumulation of cellular mutations over time and consequent progression to cancer[4]. The high-risk E5 protein cooperates with E6 and E7 to promote hyperproliferation of infected cells and is likely to facilitate malignant progression[10]. The pathways targeted by these oncoproteins as part of the viral replication strategy are frequently disrupted in human cancers[11]and contribute directly to the development of HPV-associated cancers (FIG. 2).

From infection to tumour initiation

Failure of the immune system to clear persistent HPV infections can lead to the development of cervical cancer after several decades[12]. In precancerous lesions, most HPV genomes persist in an episomal state whereas, in many high-grade lesions, genomes are found integrated into the host chromosome. Although no apparent hotspots have been identified, HPV integration often occurs near common fragile sites, which are naturally occurring regions of genomic instability[13,14]. Over one-half of HPV 16-positive cancers and most HPV 18-positive malignancies contain integrated HPV genomes, suggesting that integration may, in some cases, contribute to malignant progression. In lesions containing HPV episomes, the viral E2 protein directly represses early gene expression as part of a mechanism to regulate copy number[15]. This activity is in addition to the role of E2 as a replication factor. Integration of viral DNA usually disrupts E2 expression, leading to the deregulated expression of early viral genes, including E6 and E7 (REFS 16,17), as well as increased proliferative capacity, a crucial step in progression to cancer[11]. E6 and E7 mRNAs expressed from integrated copies show increased stability, and integration imparts a selective growth advantage over cells that harbour only episomal copies of viral DNA[18,19]. Reintroduction of E2 into HPV-positive cancer cells containing only integrated HPV genomes results in cellular senescence[20], indicating that E6 and E7 are necessary for maintenance of the transformed phenotype[21,22]. Recent studies have suggested that an important step in HPV carcinogenesis may be the coexistence of HPV episomes with integrated copies[23]. Expression of the E1 and E2 viral replication proteins from episomes can initiate DNA replication from integrated viral origins, resulting in their amplification and the induction of chromosomal abnormalities. Replication of integrated origins also results in the activation of DNA repair and recombination systems, which increases the likelihood of acquiring cellular mutations, increased genomic instability and, eventually, malignant progression.

Transforming abilities of E6 and E7

The primary transforming activity of high-risk HPVs is provided by the E6 and E7 oncoproteins[24]. These two factors act cooperatively in the development of HPV-induced cancers, with the action of one factor complementing that of the other. Both E6 and E7 are small proteins, approximately 18 and 13 kDa in size, respectively, localized in the nucleus. The E6 proteins are also found in the cytoplasm and some studies have suggested that E7 also has a cytoplasmic component[25,26]. The expression of high-risk E7 proteins by themselves can immortalize human keratinocytes at a low frequency but E6 has no such activity. The combination of E6 and E7, however, is highly efficient at immortalizing most types of primary cells[27,28]. The growth of keratinocytes

Organotypic raft culture
An *in vitro* method for growing keratinocytes at an air–liquid interphase that faithfully duplicates *in vivo* epithelial differentiation.

expressing both E6 and E7 in organotypic raft cultures, which faithfully duplicate epithelial stratification and differentiation, results in cellular changes identical to those observed in high-grade squamous intraepithelial lesions *in vivo*[29], further demonstrating the importance of these factors in HPV pathogenesis. In addition, transgenic mice that express high-risk E6 and E7 in basal epithelial cells develop squamous carcinomas on low-dose oestrogen treatment[30]. In this transgenic mouse model, E7 alone is sufficient to induce high-grade cervical dysplasia and invasive cervical malignancies. The addition of E6 results in larger and more extensive cervical cancers[31], highlighting the cooperative activity of E6 and E7 in promoting tumorigenesis.

Although the combination of E6 and E7 can efficiently immortalize cells in culture, HPV-immortalized cells are not tumorigenic in nude mouse models[32] and require extensive passaging in tissue culture or the co-expression of additional oncogenes, such as *v-ras* or *v-fos*, to acquire the ability to form tumours[33,34]. This difference compared with transgenic mouse models in which E6 and E7 efficiently induce tumours underscores the differences in the ability to transform mouse versus human cells and highlights the importance of oestrogen in the mouse model. As most people infected with genital HPVs do not develop genital cancers, expression of E6 and E7 is necessary but not sufficient for malignant progression. In the productive phase of the HPV life cycle, E6 and E7 promote the proliferation of undifferentiated and differentiated suprabasal cells and also allow avoidance of apoptosis. This leads to the accumulation of DNA damage and mutations that can result in transformation and the development of carcinomas. To understand how the cooperative action of these factors leads to cancer, it is useful to examine the multiple pathways targeted by these proteins.

Box 1 | **Human papillomavirus-associated cancers and vaccines**

Only a fraction of women infected with high-risk human papillomaviruses (HPVs) develop cervical cancer, indicating that additional factors must contribute to malignant progression[85]. Persistent infection with a high-risk HPV type is the major risk factor for the development of HPV-associated cancers. The key determinants for the development of a persistent HPV infection are age of onset of sexual activity, number of sexual partners and immune status. Two vaccines were recently introduced that block initial infection by some high-risk HPV types: Gardasil (Merck) and Cervarix (GlaxoSmithKline) both protect against infection by two high-risk types, HPVs 16 and 18. Gardasil is also directed against two low-risk types, HPVs 6 and 11. HPVs 16 and 18 are responsible for approximately 70% of cervical cancers and HPVs 6 and 11 cause approximately 90% of genital warts. Over a dozen additional HPV types cause the other 30% of HPV-associated cancers and these are not targeted by either vaccine. Although these vaccines block initial infection by certain HPV types, they do not prevent the progression to cervical cancer in individuals who are already infected. Both vaccines seem to be highly effective in blocking infections by high-risk HPVs 16 and 18, as well as the development of cervical neoplasias over a 5-year period[149]. These vaccines have recently been approved for use in men to prevent the development of HPV-associated penile cancers and should also be effective in the prevention of oral cancers associated with high-risk HPVs. It is important for women to continue to undergo Papanicolau smear screening, as infections with other high-risk HPV types not targeted by the vaccines can still occur. The development of these vaccines is a substantial advance in preventing cervical cancer worldwide and future advances can only make them more effective.

Maintenance of proliferative ability

Degradation of Rb and activation of E2F-dependent promoters. E7 proteins do not possess any intrinsic enzymatic or DNA-binding activities but function by binding to several cellular factors (FIG. 3). The best characterized of these interactions is with the RB tumour suppressor and also with the related family members p107 and p130. E7 interacts with Rb family members through a conserved LXCXE motif that is present in its amino-terminus[9]. The Rb family of 'pocket proteins' controls the G1–S phase transition by regulating the activity of the E2F family of transcription factors[35]. The E2F family consists of at least eight members, some of which act as transcriptional activators and others function as repressors. E2F1–E2F5 contain binding domains for pocket proteins but E2F6–E2F8 lack these residues and therefore regulate gene expression independently of Rb family members[36]. E2F-binding sites are found in the promoters of many genes that are involved in regulating cell cycle progression, differentiation, mitosis and apoptosis[37]. In normal cells, RB represses transcription of E2F-dependent promoters by directly binding to the E2F transactivation domain and recruiting various chromatin modifiers such as histone deacetylases (HDACs) to promoter sequences[38]. In late G1, RB is phosphorylated by cyclin-dependent kinase (CDK) complexes, which results in the disassociation of RB from E2F, leading to transcription of S phase-specific genes[39]. The binding of high-risk E7 to RB disrupts RB–E2F complexes[40], resulting in the constitutive expression of E2F-responsive genes, such as cyclin A and cyclin E, and promotes premature S phase entry and DNA synthesis[6,41]. E7 also affects the expression of S phase genes by directly interacting with E2F factors and with HDACs[42–44]. E7 binds to E2F6 (REF. 45), which acts as a transcriptional repressor by recruiting polycomb group (PcG) complexes to E2F-responsive promoters. The E7–E2F6 interaction is thought to prevent repression by E2F6–PcG complexes, maintaining an S phase environment conducive for viral replication. High-risk E7 proteins bind to HDACs through sequences distinct from those with which they bind to RB and can target HDACs to repress transcription and facilitate HDAC removal at other promoters to activate transcription[43,44,46,47]. The E7–RB–HDAC interaction is essential for episomal maintenance and for maintaining an S phase environment on differentiation, and is therefore necessary for productive viral replication in suprabasal cells[43,47]. Not only do E7 proteins form complexes with Rb family members but they also target them for proteasomal degradation through the ubiquitin-dependent pathway[48,49]. This degradation means that a low level of E7 is sufficient to prevent the association of RB, p107 or p130 with E2F members, and also abrogates other Rb activities, such as DNA repair and the maintenance of genomic integrity.

Modulation of cyclins and CDK inhibitors. In addition to RB destabilization, E7 contributes to immortalization through interaction with key proteins that control cell cycle progression. The CDK inhibitors p21 and p27 are important regulators of growth arrest during epithelial differentiation, and p21 is thought to act as a tumour suppressor in cervical carcinogenesis[50]. The major target

HECT family
A group of related E3 ubiquitin protein ligases that contain a conserved C-terminal 350 amino acid long homologous to the E6 C-terminus (HECT) domain that is involved in ubiquitylation of bound substrates.

of p21 and p27 in human keratinocytes is CDK2, which is important for G1 to S phase entry and progression through interaction with cyclin E and cyclin A, respectively[51]. E7 proteins have many strategies to maintain high CDK2 activity. The carboxy-termini of high-risk E7 proteins bind p21 and p27, efficiently neutralizing the inhibitory effects on cyclin E- and cyclin A-associated kinase activities[52,53]. In turn, CDK2 activity remains high in E7-expressing cells despite high levels of p21 (REFS 52,54). Low-risk E7 proteins can also bind p21 but with a greatly reduced efficiency and a decreased ability to abrogate the inhibitory effects of p21. High-risk and low-risk HPV E7 can bind indirectly to cyclin E and cyclin A–CDK2 complexes through RB, p107 or p130, as well as directly to CDK2 and/or cyclin subunits, allowing sustained CDK2 activity[55,56]. High-risk E7 has further been shown to increase the levels of the CDC25A phosphatase[57,58], which can induce tyrosine dephosphorylation of CDK2, promoting its activation.[59].

Abrogation of growth arrest through degradation of p53. One major consequence of the efficient targeting of RB–E2F and other cell cycle regulators by high-risk E7 proteins is an increase in the levels of the tumour suppressor p53 (REF. 60), which impairs growth and increases the susceptibility of E7-expressing cells to apoptosis[49,61]. To counteract this, high-risk E6 proteins use several mechanisms to interfere with p53 functions (FIG. 4). E6 proteins recruit the cellular E3 ubiquitin ligase E6-associated protein (E6AP), a prototypical member of the HECT family (homologous to the E6AP carboxyl-terminus family), to a trimeric complex with p53 (REF. 62), which leads to the ubiquitylation and proteasomal degradation of p53 (REFS 63,64). E6 proteins can also bind directly to p53 and block transcription by interfering with its DNA-binding activity[65]. Low-risk E6 proteins can also associate with E6AP[66] but surprisingly this does not result in p53 degradation[67], suggesting that other cellular factors are targets for the low-risk E6–E6AP complex.

The degradation of p53 by the E6–E6AP complex reduces the net levels of p53 but remaining p53 can be activated in response to DNA damage and other cellular stresses. E6 also interferes with p53 function by binding to the two related histone acetyltransferases p300 and CREB-binding protein (CBP), blocking the ability of these factors to acetylate p53 and therefore increase its stability[68,69]. E6 proteins also bind to the histone acetyltransferase ADA3, which can similarly affect p53 activity[70]. Interestingly, in contrast to p300 and CBP, E6 inactivates ADA3 by targeting it for degradation. Low-risk HPV E6 proteins also inhibit the transcriptional activity of p53 through direct binding[65,71] and this may be the predominant mechanism by which low-risk HPVs inhibit the growth-suppressive affects of p53. The primary reason that high-risk E6 proteins block p53 function is to facilitate productive viral replication but this in turn has consequences for tumour development. As HPV infections persist for extended periods, the abrogation of p53 function allows genetic mutations to accumulate that normally would have been repaired. Interestingly, E6 does not bind to or promote the degradation of the p53 homologues, p73 and p63 (REFS 72,73), indicating that the inactivation of these proteins is not necessary for transformation.

Although the effects of high-risk E6 on p53 are central to the development of genital cancers, additional p53-independent targets play equally important parts. E6 mutants deficient for degradation of p53 can still immortalize cells[74,75], suggesting that interactions with other cellular factors are necessary for cancer development. Among the important p53-independent targets are the PDZ proteins that associate only with high-risk E6 proteins[76]. Mutation of the PDZ-binding domain of E6 in the context of complete viral genomes leads to reduced growth rates, loss of viral episomes and frequent integration of viral genomes into host chromosomes, indicating the importance of these interactions for viral pathogenesis[77]. Importantly, transgenic mice encoding E6 proteins defective for binding to PDZ partners do not develop hyperplasia or tumours[78].

In transient overexpression assays, E6 has also been shown to bind to a series of other factors that may also contribute to transformation. Among these are E6-binding protein (E6BP, also known as reticulocalbin 2), a calcium-binding protein found in the endoplasmic reticulum; E6-targeted protein 1 (E6TP1, also known as SIPA1-like protein 1), a GTPase–activating protein; and minichromosome maintenance 7 (MCM7), a regulator of replication[26]. It is not clear, however, which of these E6 activities are important *in vivo*.

Immortalization through the activation of telomerase. For cells to become immortal they must induce the expression of telomerase, an enzyme often activated in cancers that is important for replicating the DNA

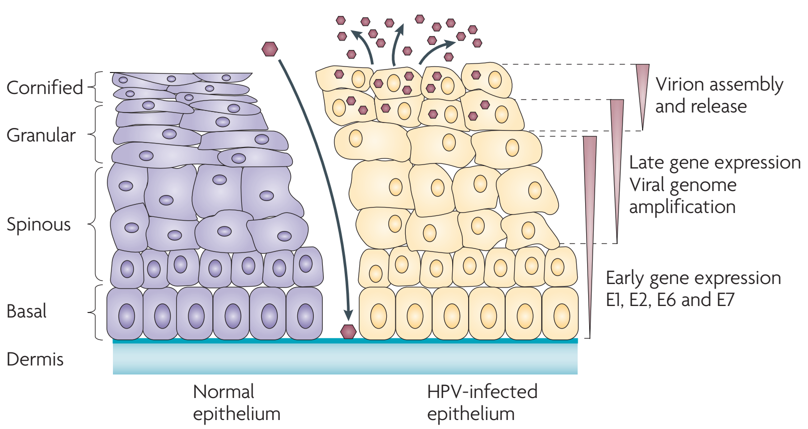

Figure 1 | **The life cycle of human papillomaviruses.** Human papillomaviruses (HPVs) infect keratinocytes in the basal layer of the epithelium that becomes exposed through microwounds. Uninfected epithelium is shown on the left and HPV-infected epithelium is shown on the right. On infection, the viral genomes are established in the nucleus as low-copy episomes and early viral genes are expressed. The viral genomes are replicated in synchrony with cellular DNA replication. After cell division, one daughter cell migrates away from the basal layer and undergoes differentiation. Differentiation of HPV-positive cells induces the productive phase of the viral life cycle, which requires cellular DNA synthesis machinery. The expression of E6 and E7 deregulates cell cycle control, pushing differentiating cells into S phase, allowing viral genome amplification in cells that normally would have exited the cell cycle. The late-phase L1 and L2 proteins encapsidate newly synthesized viral genomes and virions are shed from the uppermost layers of the epithelium (red hexagons).

Telomere
A double-stranded short tandem repeat found at the ends of chromosomes that consists of the sequence TTAGGG and is approximately 10–15 kb in length. Telomeres provide a cap for linear chromosomes and are important in maintaining genomic stability.

Telomerase reverse transcriptase
The catalytic protein subunit of telomerase, an RNA-dependent DNA polymerase that synthesizes telomere repeats at chromosomal ends.

Centrosome
The primary microtubule-organizing centre of human cells, which consists of a pair of centrioles. Centrosomes are duplicated only once before mitosis and are responsible for proper chromosome segregation during cell division.

sequences at the ends of chromosomes called telomeres[79]. High-risk E6 proteins activate transcription of telomerase reverse transcriptase (TERT), which along with RB inactivation by E7, is an essential step in immortalization[26,80]. E6 activates TERT and telomerase through E6AP by interacting with MYC and modulating the activity of repressors (upstream stimulating factors 1 and 2 (USF1 and USF2) and nuclear transcription factor, X box-binding protein 1-91(NFX1-91)) and activators (MYC–MAX, SP1 and histone acetyltransferases) that bind to the *TERT* promoter[26]. E6 also directly associates with NFX123 to increase TERT levels through transcriptional and post-transcriptional mechanisms[81]. A more detailed discussion of the mechanisms by which E6 affects telomerase activation is provided in a recent review by Howie *et al.*[26]. Interestingly, E7 can promote telomere maintenance in the absence of E6 even when minimal telomerase activation is detected[82]. Some studies have suggested that E7 promotes telomere lengthening through the alternative lengthening of telomeres (ALT) pathway, which involves homologous recombination between telomere sister chromatids[83,84]. Although high levels of telomerase are detected in most cervical cancers, some early cervical lesions lack detectable TERT expression, yet can persist for long periods of time despite having shortened telomeres[83]. It is possible that the activation of ALT by E7 is important in maintaining telomere length early in cancer development to reduce genomic instability and promote tumour progression. This would allow the clonal outgrowth of cells that maintain a minimal level of telomerase activity, and is consistent with the notion that E6 plays a part in tumour progression by primarily promoting telomerase activity in high-grade cervical lesions and carcinomas.

Genomic instability

Although E6 and E7 are necessary for maintenance of the transformed phenotype, they are not sufficient to directly transform cells. Additional oncogenic events such as genomic instability are necessary for malignant progression to occur. This is consistent with the long latency period between initial HPV infection and the development of cancer[85]. High-risk E6 and E7 independently induce genomic instability in normal cells[86], which is a characteristic of high-risk HPV-induced malignancies. Most HPV-associated malignancies have numerous chromosomal imbalances, including gains or losses of whole chromosomes (aneuploidy) and chromosomal rearrangements[87]. Induction of genetic instability is thought to be an early event in HPV-induced cancers, occurring before integration of the virus into host chromosomes[88]. Consistent with this notion, aneuploidy can be detected in pre-malignant HPV-associated cervical lesions[89,90]. These activities are limited to high-risk E6 and E7 proteins as no such activities are seen in cells expressing their low-risk counterparts[91].

Centrosome abnormalities. The expression of high-risk E6 and E7 quickly induces numerous mitotic defects, including multipolar mitoses, anaphase bridges and aneuploidy[92]. Abnormal multipolar mitoses are

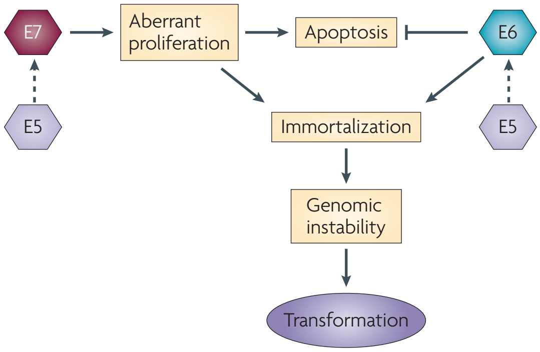

Figure 2 | **Molecular mechanisms by which the human papillomavirus oncoproteins cooperate to induce cervical carcinogenesis.** The induction of hyperproliferation by the E7 protein triggers apoptosis, which is blocked by the actions of the E6 protein. The cooperative actions of E6 and E7 efficiently immortalize cells and this process is augmented by the actions of the E5 protein. The ability of E6 and E7 to target crucial regulators of proliferation, apoptosis, immortalization and genomic stability collectively promotes the emergence of a clonal population of cells with a growth advantage and an increased propensity for transformation and malignant progression.

characteristic of most high-risk HPV lesions and are associated with abnormal centrosome numbers[93,94]. E6 and E7 cooperate to induce centrosome abnormalities[94], which can lead to chromosomal missegregation and the development of aneuploidy. In E6-expressing cells, aberrant centrosome numbers occur concomitantly with nuclear atypia and only become evident after prolonged passaging. By contrast, E7 rapidly induces centrosome amplification, which correlates with cell division errors and occurs before the detection of genomic instability[88]. These studies suggest that aberrant centrosome duplication is an early event that may drive chromosomal instability. E7 induces multiple rounds of centrosome synthesis in a single S phase through the formation of multiple immature centrioles from a single maternal centriole[95]. In contrast to normal centrosome duplication, E7-mediated centrosome amplification is dependent on high levels of CDK2 activity[96], linking this function of E7 to the degradation of Rb family members. Although the E7 LXCXE Rb-binding motif is necessary for aberrant centrosome synthesis, E7 can induce centrosome abnormalities in mouse embryonic fibroblasts deficient in Rb family members, albeit at a much reduced level[97]. This may be due to the binding of E7 to γ-tubulin, a centrosome regulator, through sequences that overlap with the Rb-binding domain[98]. This interaction is independent of Rb binding and results in removal of γ-tubulin from the mitotic spindle, which may result in abnormal centrosome synthesis.

Although cells with abnormal mitoses are normally targeted for cell death, E6 and E7 act cooperatively to allow cells with abnormal centrosomes to accumulate, possibly by relaxing the G2–M checkpoint response that is normally regulated by p53 (REF. 99) and also through inhibition of apoptotic signalling[100]. In addition to

ATM–ATR pathway
This involves phosphoinositide 3-like kinases important in sensing and repairing DNA damage. ATM is activated in response to double-stranded breaks and ATR is induced on the appearance of single-stranded lesions.

centrosome abnormalities, E6 and E7 have been shown to independently bypass mitotic checkpoints[101,102], resulting in the accumulation of polyploid cells that can lead to aneuploidy. Abrogation of these checkpoints may be important for viral replication but can also lead to genomic instability in HPV-immortalized cells. As malignant progression occurs over many years, it is likely that these mitotic defects occur infrequently and do not often lead to viable progeny. The accumulation of subtle chromosomal alterations may provide a growth advantage to a subclone of HPV-positive cells, resulting in the outgrowth of a cellular population that contributes to viral persistence and, ultimately, malignant progression.

DNA damage. E6 and E7 can also induce genomic instability through the induction of DNA damage and the activation of the ATM–ATR pathway (ataxia telangiectasia-mutated–ATM and RAD3-related DNA damage repair pathway). E6 and E7 have been shown to independently induce DNA damage[103] and increase the frequency of foreign DNA integration into the host genome[104]. High-risk E7 has been shown to activate the ATM pathway in undifferentiated and differentiated keratinocytes[105]. ATM and its downstream target CHK2 are activated in undifferentiated and differentiated HPV-positive cells, but, a crucial member of this pathway, Nijmegen breakage syndrome 1 (NBS1, also known as nibrin), is only activated in differentiated cells. Nuclear foci containing histone γ-H2AX, a modified histone specific to DNA repair, are induced by HPV and may reflect the localization of DNA repair proteins to replication foci. Through the use of inhibitors, the activation of the ATM DNA damage response was shown to be important for differentiation-dependent viral genome amplification but not the stable maintenance of episomes in undifferentiated cells[105]. ATM and ATR can also be activated after the replication of integrated copies of HPV genomes, which leads to the amplification of integrated HPV sequences and the flanking cellular sequences[23]. Activation of the DNA damage response in cells containing both episomal and integrated forms of the viral genome could therefore result in chromosomal alterations and induction of genomic instability, which are likely to be important in the progression to malignancy.

An important aspect of the ATM–ATR DNA damage response is the induction of cell cycle checkpoints at S or G2–M, and E7 can abrogate these checkpoints to promote mitotic entry. E7 induces the degradation of claspin[106], a key regulator of the ATR–CHK1 DNA damage-signalling pathway that is activated in response to replication stress. Claspin proteolysis is essential for DNA damage checkpoint recovery[107]. The accelerated degradation of claspin by E7 in G2–M may 'trick' cells into initiating checkpoint recovery, allowing aberrant mitotic entry in the presence of DNA damage, potentially

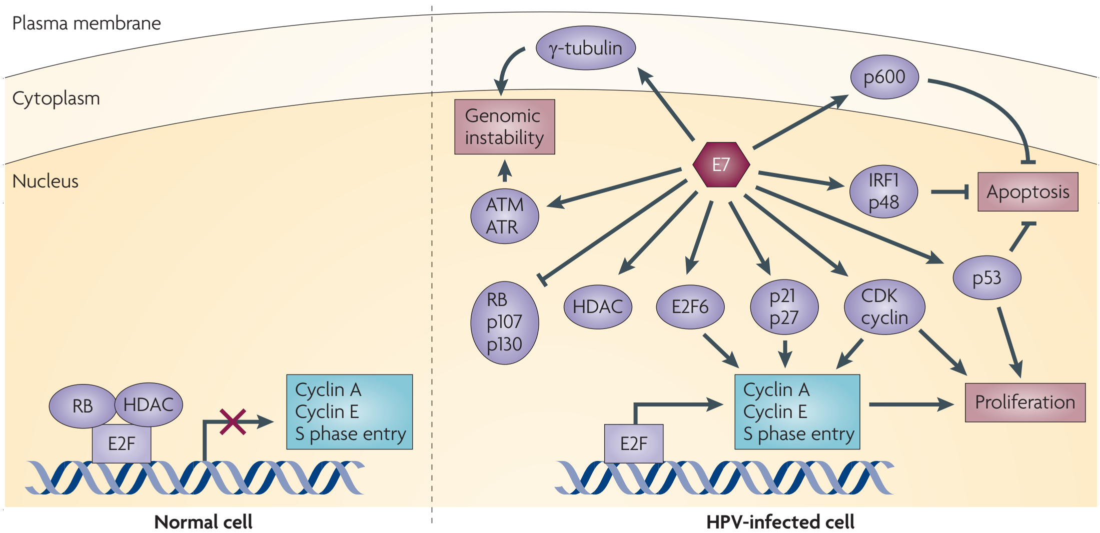

Figure 3 | **The human papillomavirus E7 oncoprotein affects numerous cellular processes through interactions with multiple host cell proteins.** High-risk human papillomavirus (HPV) E7 proteins subvert G1–S arrest and induce hyperproliferation through inhibition of retinoblastoma (RB) family members and constitutive activation of E2F-responsive genes. E7 also affects cellular gene expression through interaction with histone deacetylases (HDACs) and E2F6. E7 further deregulates cell cycle control through inhibition of cyclin-dependent kinase inhibitors (such as p21 and p27), stimulation of cyclins and through direct activation of cyclin-dependent kinase 2 (CDK2). E7 stimulates abnormal centrosome synthesis through increased CDK2 activity and by interacting with γ-tubulin, leading to an increased risk of genomic instability. E7 induces DNA damage and activation of the ATM–ATR pathway (ataxia telangiectasia-mutated–ATM and RAD3-related DNA damage response) which may contribute to the accumulation of chromosomal alterations. Co-expression of HPV E6 with E7 abrogates p53-dependent apoptosis in response to the activities of E7, allowing replication in the presence of DNA damage and increased chromosomal instability. The interaction of E7 with p600 prevents anoikis and allows anchorage-independent growth, promoting malignant progression. E7 interacts with components of the interferon (IFN) response (IFN regulatory factor 1 (IRF1) and p48), contributing to escape from immune surveillance and the establishment of a persistent infection.

Fanconi anaemia
A rare disease characterized by chromosomal instability and a high incidence of squamous cell carcinomas of the head, neck and anogenital regions.

Anoikis
A form of programmed cell death that is activated when normal cells attempt to divide in the absence of attachment to the extracellular matrix.

leading to genomic instability through defective DNA repair. The activation of DNA damage pathways by HPV proteins is necessary for productive viral replication but this in turn contributes to malignant progression. HPV has also recently been linked to the Fanconi anaemia (FA) pathway[12,108], which promotes DNA repair in response to replication stress. HPV 16 E7 normally activates the FA pathway and, in cells deficient for an intact FA response, E7 expression leads to increased chromosomal instability[109].

Apoptosis

HPV proteins can also extend the proliferative capacity of infected cells by blocking apoptosis. Induction of aberrant proliferation and/or DNA synthesis in the absence of sufficient growth signals, such as that which occurs in differentiating HPV-positive cells, results in a p53-dependent apoptosis termed the tropic sentinel response[110]. The abrogation of Rb functions by high-risk E7 proteins sensitizes cells to p53-dependent apoptosis and this is blocked by E6 (REF. 61). Targeting of p53 for degradation, inhibition of p53 acetylation and suppression of p53 target gene expression compose only a subset of the mechanisms by which E6 and E7 counteract the various stimuli that can induce programmed cell death. Interestingly, recent studies indicate that HPV uses apoptotic signalling pathways to promote replication in differentiating cells[111] (BOX 2).

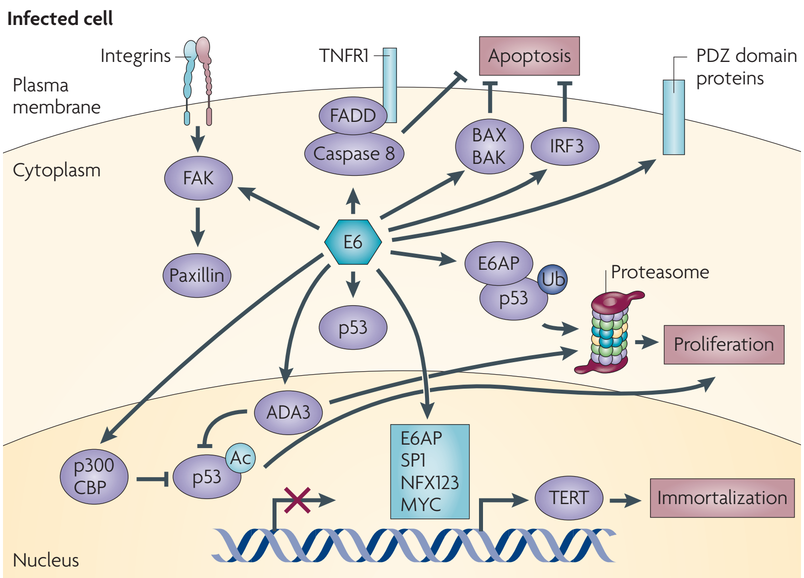

Figure 4 | **Cellular proteins and signalling pathways affected by the human papillomavirus E6 oncoprotein.** High-risk E6 proteins inhibit p53-dependent growth arrest and apoptosis in response to aberrant proliferation through several mechanisms, resulting in the induction of genomic instability and the accumulation of cellular mutations. Formation of an E6–E6-associated protein (E6AP)–p53 trimeric complex results in p53 degradation, and the interaction of E6 with the histone acetyltransferases p300, CREB binding protein (CBP) and ADA3 prevents p53 acetylation (Ac), inhibiting the transcription of p53-responsive genes. E6 also inhibits apoptotic signalling in response to growth-suppressive cytokines through interaction with the tumour necrosis factor (TNF)-α receptor TNFR1, FAS-associated protein with death domain (FADD) and caspase 8, and through the degradation of pro-apoptotic BAX and BAK. The interaction of E6 with SP1, MYC, nuclear transcription factor, X box-binding protein-123 (NFX123) and E6AP activates telomerase reverse transcriptase (TERT) and telomerase, preventing telomere shortening in response to persistent proliferation and in turn promoting immortalization. E6-mediated degradation of PDZ proteins leads to loss of cell polarity and induces hyperplasia. The interaction of E6 with the focal adhesion protein paxillin and the extracellular matrix protein fibulin prevents anoikis and allows cellular growth in the absence of attachment to extracellular matrix. E6 subverts the interferon (IFN) response through interaction with IFN regulatory factor 3 (IRF3) and through the inhibition of p53 activity. FAK, focal adhesion kinase; Ub, ubiquitin.

Inhibition of anoikis. Another major apoptotic pathway targeted by HPV proteins is anoikis, which is associated with anchorage-independent growth[112]. Integrins interact with the extracellular matrix (ECM), and regulate signal transduction through focal adhesion kinase (FAK). Adhesion to the ECM results in the phosphorylation and activation of FAK and its downstream substrate paxillin, which leads to cytoskeletal reorganization and formation of focal adhesions. HPV-positive cells express high levels of FAK and fibronectin and have increased phosphorylation of paxillin[113]. The bovine papillomavirus (BPV) E6 protein binds paxillin, which correlates with its transformation function[114,115]. Although HPV 16 E6 has also been reported to bind to paxillin, it is unclear how this interaction contributes to pathogenesis[114,115]. HPV 16 E6 also binds to the ECM protein fibulin 1 (REF. 116), which plays a part in transformation and tumour invasion. These interactions, coupled with FAK activation, promote resistance to anoikis and allow HPV-immortalized cells to proliferate in the absence of adherence to the ECM. E7 also disrupts anoikis through interaction with the RB-associated protein p600 that functions as an ubiquitin ligase[117]. p600 is a cytoplasmic protein, suggesting that E7 can target cellular factors in the cytoplasm and the nucleus. Binding to p600 is mediated through N-terminal sequences of E7 and deletion of these sequences abrogates transformation in an RB-independent manner. Furthermore, the depletion of p600 in HPV-positive cancer cells results in impaired anchorage-independent cell growth, suggesting that p600 is important for cellular transformation, as well as prevention of anoikis.

Resistance to growth-suppressive cytokines. E6 and E7 also interfere with the effects of various growth inhibitory cytokines that are induced following infection. In response to viral entry, cells produce inflammatory mediators such as tumour necrosis factor–α (TNFα), which is a potent inhibitor of keratinocyte proliferation[118]. Inflammatory cytokines can activate the extrinsic apoptotic pathway through transmembrane cell surface death receptors of the TNF receptor family, such as TNF receptor 1 (TNFR1), FAS (also known as CD95) and the TNF-related apoptosis-inducing ligand (TRAIL) receptors. High-risk E6 proteins block apoptosis induced by TNFα by directly binding to TNFR1, which inhibits the formation of the death-inducing signalling complex and consequent transduction of apoptotic signals[119]. E6 also interacts with the adaptor protein FAS-associated protein with death domain (FADD) and caspase 8 to block cell death in response to FAS and TRAIL[120,121]. E6 can also interfere with induction of the extrinsic and intrinsic (mitochondrial) apoptotic pathways through interactions

Box 2 | **Caspase activation in human papillomavirus pathogenesis**

Although E6 and E7 act cooperatively to block apoptosis in undifferentiated cells, they induce low levels of caspase activation in differentiating cells, which are important for productive viral replication[111]. One of the targets of differentiation-dependent caspase activation is the E1 replication protein that acts as an origin recognition factor and helicase. E1 proteins are cleaved by caspases 3 and 7 following differentiation to presumably generate a more active replication factor. The lack of apoptosis in these differentiating cells is the result of low levels of caspase activation, coupled with an increase in anti-apoptotic proteins, such as BCL2 (REF. 111). Human papillomavirus (HPV) infections induce ataxia telangiectasia-mutated (ATM) and CHK2 activation in differentiating keratinocytes. Caspase activation is abrogated in the presence of a CHK2 inhibitor, indicating these two pathways are linked and important for HPV genome amplification[105]. It is intriguing that E6 and E7 can block apoptosis in undifferentiated cells but activate low levels of caspase cleavage in differentiated cells.

with the pro-apoptotic Bcl2 members BAK and BAX, as well as by upregulation of inhibitors of apoptosis such as inhibitor of apoptosis protein 2 (IAP2, also known as BIRC2) and survivin (also known as BIRC5)[122]. These activities of E6 are necessary for continued proliferation in the presence of pro-apoptotic signals and it is unclear if low-risk E6 proteins function in a similar manner.

Subversion of the interferon antiviral response. Interferon (IFN) is activated following viral infection and HPV proteins act at several levels to interfere with this response[123]. Hundreds of cellular genes are induced on exposure to IFN and microarray studies have shown that high-risk HPV proteins repress the transcription of many IFN-inducible genes, including myxovirus resistance protein A (MXA, also known as MX1), 2′-5′-oligoadenylate synthetase 2 (OAS2) and signal transducer and activator of transcription 1 (STAT1)[124]. STAT1 is a key transcription factor that regulates the IFN response and its repression by E6 and E7 may be crucial for the inhibition of this activity. Other studies have shown that E6 directly interacts with components of the IFN response, such as IFN regulatory factor 3 (IRF3), which inhibits transactivation of IFN transcription[125]. HPV 16 E7 also binds IRF1 (REF. 126) and p48 (REF. 127), a component of the IFN-stimulated gene factor 3 complex, which blocks STAT1–STAT2 heterodimer translocation into the nucleus in response to IFN. High-risk E6 and E7 proteins also target other members of the IFN pathway, such as the double-stranded RNA protein kinase PKR, which inhibits protein synthesis through phosphorylation of the eIF2a proteins[128,129]. E6 blocks PKR kinase activity by relocalizing it to cytoplasmic P-bodies, which are sites of mRNA storage and degradation, although the mechanism by which this occurs is still not clear[128].

p53 is not only important for controlling cell cycle progression in response to genotoxic stress but also in mediating the antiproliferative effects of the IFN response. Treatment of HPV 16 E7-expressing keratinocytes with IFN results in senescence that is dependent on p53 acetylation by the histone acetyltransferases p300 and CBP[130]. E6 overcomes the growth-suppressive effects of IFN independently of E6AP and p53 degradation through the formation of a tripartite complex with p53 and p300/CBP. This interaction prevents the acetylation of p53 and blocks the activation of p53-target genes, allowing continued viral replication in the presence of IFN[130]. Importantly, the binding of E6 to p300 or CBP may facilitate viral escape from immune surveillance and establish long-term persistence.

Cooperative transforming activities of E5

Although E6 and E7 provide the primary transforming activities of high-risk HPV viruses, E5 can augment their function and contribute to tumour progression[10] (FIG. 5). The high-risk E5 proteins are small, hydrophobic peptides, approximately 83 amino acids in size that localize primarily to the endoplasmic reticulum[131–133]. When expressed alone, HPV E5 has weak transforming activity, in contrast to its bovine counterpart, BPV1 E5, which shows strong transforming activity[134]. In tissue culture assays, HPV E5 can enhance the transforming activity of E6 and E7 (REFS 135–137), suggesting that it may have a supportive role in tumour progression. Importantly, in transgenic mouse models, high-level expression of HPV 16 E5 in the skin induces epithelial hyperproliferation that results in spontaneous tumour formation[138]. In oestrogen-treated mice, expression of E5 alone can induce cervical cancers[139], suggesting that — in some cases — E5 functions as an oncogene. In many cases, HPV 16-positive cervical tumours contain viral episomes in addition to viral integrants[140–142]. It is therefore possible that there are multiple paths to HPV-induced tumorigenesis. One way is through high-level

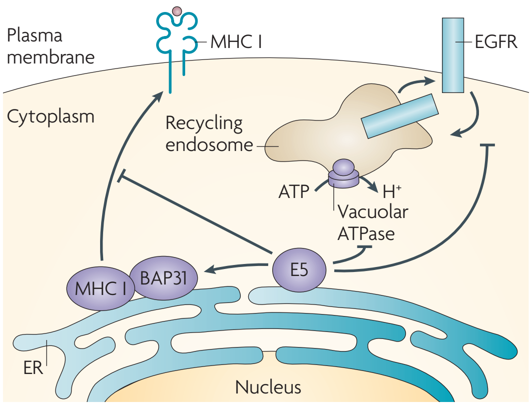

Figure 5 | **High-risk E5 interactions with cellular pathways and factors.** E5 contributes to the actions of E6 and E7 by modulating the transit of signalling proteins through the endoplasmic reticulum (ER) as well as by interacting with factors such as B cell receptor-associated protein 31 (BAP31) and the vacuolar H^+-ATPase in endosomes. E5 expression results in increased epidermal growth factor receptor (EGFR) signalling and activation of the MAPK pathway, which augments the activities of E6 and E7, resulting in aberrant proliferation. The interaction of E5 with the vacuolar H^+-ATPase may promote recycling of receptors to the cell surface by impairing organelle acidification, resulting in constitutive signalling. E5 has been reported to reduce levels of major histocompatibility complex class I (MHC I) at the cell surface, which may occur through its interaction with the ER protein BAP31, and prevent clearance of infected cells by the immune response.

expression of E6 and E7 owing to viral integration into host chromosomes and consequent abrogation of the repressive effects of E2. A second path could occur in cells that still maintain viral episomes, in which the expression of E1 and E2 promotes genomic instability through the aberrant replication of integrated HPV sequences. In addition, the expression of E5 would augment the activity of E6 and E7, resulting in tumour progression (FIG. 2).

The expression of E5 is increased on differentiation to promote proliferation of differentiated cells and productive viral replication. The localization of HPV E5 to the endoplasmic reticulum suggests its activity may be related to the trafficking of cytoplasmic membrane proteins through this cellular compartment. This is consistent with the identification of B cell receptor-associated protein 31 (BAP31), a regulator of membrane protein transport, as a binding partner of E5 (REF. 143). E5 has also been reported to alter the activity of the epidermal growth factor receptor (EGFR, also known as ERBB1)[144,145], which may occur through its binding to the vacuolar ATPase, resulting in altered endosomal pH[131] and therefore interfering with EGFR turnover. Other activities attributed to E5 include reducing the surface levels of major histocompatibility complex (MHC) class I proteins[146], modulating the MAPK pathway[147] and altering the levels of caveolin 1 (REF. 148). The role of the E5 protein in tumour progression is intriguing and an area of active study.

Perspective

The development of cervical cancers involves a coordinated targeting of multiple pathways by HPV oncoproteins, with each pathway having a distinct role in malignant progression. HPV proteins disrupt or usurp multiple cellular signalling pathways to maintain infected cells in a proliferative state to facilitate viral replication and persistence. One consequence of this, however, is the accumulation of mutations in cellular genes and increased genomic instability, which results in full transformation. The primary viral factors responsible for altering these pathways and mediating progression to malignancy are the E5, E6 and E7 proteins. The efficient disruption of p53 and Rb function by E6 and E7 is crucial for this process but recent studies have identified other equally important cellular targets, including telomerase, members of the DNA damage pathway, factors important for centrosome duplication and signalling proteins. By targeting multiple pathways, HPV promotes cell proliferation in the face of activated cellular defences. A new appreciation of the role of other viral proteins such as E5 in progression of HPV-induced disease is also emerging. Although recently introduced vaccines are effective in preventing initial infections by high-risk HPV types, they have no effect on existing HPV lesions and cancers. Understanding the various cellular pathways altered by HPV and stimulation of immune surveillance provides opportunities for effective therapeutic approaches against these devastating cancers.

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Acknowledgements
L.A.L. is supported by grants from the National Cancer Institute and the National Institute for Allergy and Infectious Diseases. C.A.M. is supported by a K99 Pathway to Independence Award from the National Cancer Institute. We thank K. Simanis for assistance with the figures.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
γ-H2AX | ADA3 | BAK | BAP31 | BAX | caveolin 1 | CBP | CDC25A | CDK2 | CHK1 | CHK2 | claspin | E2F6 | E6AP | E6BP | E6TP1 | EGFR | FADD | FAK | FAS | fibronectin | fibulin 1 | IAP2 | IRF1 | IRF3 | MAX | MCM7 | MXA | MYC | OAS2 | p21 | p27 | p48 | p53 | p105 | p107 | p130 | p300 | p600 | paxillin | PKR | SP1 | STAT1 | STAT2 | survivin | TERT | TNFα | TNFR1 | USF1 | USF2

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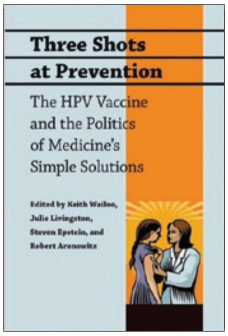
Vaccine politics



Three Shots at Prevention: the HPV Vaccine and the Politics of Medicine's Simple Solutions

Edited by Keith Wailoo, Julie Livingston, Steven Epstein and Robert Aronowitz

The Johns Hopkins University Press, 2010
352 pp., hardcover, $65.00
ISBN: 9780801896712

Reviewed by Arthur Allen

Mass vaccination campaigns against infectious disease involve a tangle of cultural, political and medical issues, whose impact varies across time and place. When Cotton Mather argued for smallpox vaccination in Boston in 1721, the city was in the throes of an epidemic of the disease, which had already killed a fifth of the city's population. Mather's notion that scratching smallpox pus into healthy individuals would protect them from the scourge provoked a storm of revulsion and anger. Yet the threat it addressed was immediate and obvious.

By contrast, when Merck began advertising its human papilloma virus vaccine, Gardasil, in 2005, vaccination was a well-established procedure. But the company was attempting to convince parents to have their preteen girls injected with a product that would theoretically protect them three or four decades later against a disease that could already be substantially controlled by routine medical examination.

The rollout of the HPV vaccine, as described in *Three Shots at Prevention*, a useful and thought-provoking collection of 15 essays, demonstrates the complexities of vaccination science. On its face, the vaccine is an exceptional preventive. Both Gardasil and Cervarix, sold by GlaxoSmithKline, were created with ingenious bioengineered molecules that mimic HPV proteins. Both vaccines have proved safe and provide excellent protection against the two HPV strains that appear to cause two-thirds of the infections that lead to cervical cancer.

In the US, Merck took the unusual approach of beginning a heavy marketing campaign of the vaccine before Food and Drug Administration and Centers for Disease Control and Prevention advisory committees had completed their reviews and before state public health officials had decided how to incorporate the vaccine into their programs. This might have been justified if the vaccine had offered full protection against a pressing disease threat. Because it did not, the urgency with which Merck and its allies championed HPV vaccination evoked skepticism from many others, including friends.

Although the dramatic impact of infantile hepatitis B vaccination on preventing adult viral carriage offers an encouraging example, the long-term efficacy of the HPV vaccine remains unknown. Concerns have been raised that the vaccine could lead insurers to stop paying for routine pelvic exams or that the ecological niche occupied by HPV vaccine serotypes 16 and 18 could be occupied by other tumorigenic strains.

Additionally, in countries with regular gynecological screening, cervical cancer is primarily a disease of the disadvantaged and the poor, who also tend to have a lower age of sexual debut. Experience in the US had shown that the best way to protect these underserved populations from a sexually transmitted disease was by mandating a vaccine as a conidtion for school attendance. Thus, public health officials who wanted the vaccine to have an impact were put in the position of requiring parents to contemplate that their 12-year-old daughters would need a vaccine for protection during sex.

The vaccine's inventors envisioned it as a tool whose most powerful impact would be on the 200,000-plus women who die of cervical cancer every year in the developing world. Yet it was initially priced for and marketed to populations that, as a group, needed it least. The backlash against Gardasil, writes Robert Aronowitz in one of the essays here, was fueled by the apparent hypocrisy of "dressing" the vaccine as a public good while selling it as a consumer product.

A mandate for, say, the measles vaccine is legally justified to build the herd immunity required to prevent the spread of that disease to the vulnerable. This argument appears weak in the case of HPV, notes medical historian James Colgrove in his essay. First, the Gardasil campaign neglected a principal vector of the disease (males), and it was geared at least initially at protecting individual girls rather than society, with herd immunity a rather distant goal.

But Colgrove rightly notes that coercion has proved effective in public health campaigns—to get motorcyclists to use helmets and drivers to use seatbelts, for example, and that compulsory vaccination laws exercise a stabilizing influence amid "fluctuations in public trust." And although pelvic exams have dramatically reduced cervical cancer's impact on the developed world, HPV infections still lead to an estimated 11,000 cervical carcinomas and 3,800 deaths each year in the US.

Economic considerations and skepticism about the vaccine's efficacy have been the major focus during the introduction of HPV in Europe. In France, the historian Ilana Lowy notes in her essay, the vaccine has been marginalized from public health campaigns, in part by an earlier controversy over allegations that the hepatitis B vaccine had caused cases of autoimmune disease.

Purchases or mandates of the HPV vaccine in wealthier countries may eventually make it easier for Merck and GlaxoSmithKline to sell their vaccines at affordable prices in the developing world; indeed, the Global Alliance for Vaccine Initiatives is already involved in negotiating such introductions. But we aren't privy to the strategic pricing decisions of the pharmaceutical companies, and epidemiological evidence for HPV subtype prevalence in Africa is still somewhat sketchy.

We get a vivid sense of the harm these vaccines could prevent in the essay of Doreen Ramogola-Masire, a Botswanan clinician who sees many HIV-infected women dying with aggressive cervical tumors. How long will it take before they can be protected from cervical cancer? This is a question that the controversy over HPV has scarcely addressed.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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The value of HPV vaccination

Persistent infection with high-risk types of human papillomavirus (HPV) causes almost all cervical cancers. Two HPV vaccines have been licensed for prophylactic vaccination in many countries and have been highly effective at preventing infection by the vaccine-targeted HPV types. Two recent papers describe the four-year, end-of-study data from a large-scale trial of Cervarix, a vaccine that targets the most prevalent oncogenic HPV types 16 and 18, in women aged 15–25 years[1,2]. The results indicate that the vaccine has high efficacy against cervical precancer development and is cross protective against nonvaccine oncogenic HPV types. We asked four experts to comment on this trial and the implications for vaccination and cervical cancer screening policies.

Jane Kim

The PATRICIA (Papilloma Trial against Cancer in Young Adults) results[1,2] reinforce evidence from previous studies that Cervarix has tremendous potential to prevent cervical cancers associated with two vaccine-targeted types, HPV16 and HPV18, as well as some nonvaccine types through cross-protection.

The cross-protective efficacy of Cervarix is higher than that reported for a quadrivalent vaccine (Gardasil)[3] that also protects against HPV6 and HPV11. For decisions about which vaccine to adopt, important considerations include the overall burden of cervical cancer, genital warts and other HPV-related conditions; the proportion of these cases attributable to HPV types targeted or cross-protected by the vaccines; and the existence of cervical cancer screening programs. For example, the relative public health impact of HPV vaccination with the bivalent versus quadrivalent vaccine in a place such as the US that can rely on a well-established screening program to prevent the remaining cervical cancers that are caused by non-16/18 HPV types and that also spends over $220 million each year treating genital warts[4] will be different from countries that lack adequate screening programs and may wish to prioritize higher cross-protection over prevention of genital warts.

The relative value (that is, cost-effectiveness) and affordability of the two vaccines will also depend heavily on vaccine price. Despite evidence that both HPV vaccines represent good value for money across different settings, the financial requirements to implement, scale up and sustain an HPV vaccination program at current prices, ranging from $5 to $130 per dose, may be prohibitive, especially in developing countries where cervical cancer burden is greatest[5]. Promoting access to either of these vaccines will move us toward achieving effective, efficient and equitable cervical cancer prevention globally.

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COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

Douglas R Lowy

These studies add to the accumulating positive data on the safety and efficacy of the bivalent (HPV16 and HPV18) vaccine Cervarix and the quadrivalent (HPV6, HPV11, HPV16 and HPV18) vaccine Gardasil[1,2,6]. HPV16 and HPV18 cause ~70% of cervical cancer and a higher percentage of HPV-associated noncervical cancers[7]. The non-HPV16/18 cases of cervical cancer are attributable to approximately ten other HPV types. HPV6 and HPV11 cause ~90% of genital warts. Both vaccines confer almost complete protection against new genital infection and disease induced by the HPV types they target. Neither vaccine alters the course of preexisting HPV16/18 infections, and there is a high risk of developing genital HPV infection soon after sexual debut. Therefore, the vaccines are most beneficial if given before sexual debut, as confirmed by widespread introduction of Gardasil in women through to 26 years old in Australia, associated with a dramatic reduction in cervical precancer among women younger than 18, some reduction in women aged 18–20 and no reduction in women aged 21–26 (ref. 8). Both vaccines continue to protect against the targeted HPV types several years longer than the four years of the current reports, with no indication of waning protection. Their safety profile seems to be similar to that of other vaccines[9].

The studies highlight the partial cross-protection against nonvaccine HPV types phylogenetically related to HPV16 and HPV18, resulting in greater protection against all cervical precancerous development (CIN3+)

Karen K Smith-McCune

The end-of-trial results from the PATRICIA demonstrate that Cervarix provides significant overall efficacy (45.6%) against cervical intraepithelial neoplasia grade 3 and adenocarcinoma *in situ* lesions (CIN3+), the proximal precursors to cervical cancer, irrespective of associated HPV type[1]. The trial enrolled healthy women aged 15–25 years who had no more than six sexual partners and had never undergone colposcopic examination.

Notably, vaccine efficacy against CIN3+ was higher (93.2%) in the subset who at baseline were naive to infection with 14 oncogenic HPV types, were seronegative for HPV16 and HPV18 and had normal cervical cytology. This reinforces the importance of the goal of vaccinating 11- to 12-year-olds before sexual exposure to HPV. The large difference in efficacy between the total vaccinated cohort and the HPV-naive subgroup raises the possibility that the benefit of vaccination was accrued in the naive subgroup and that vaccination of HPV-exposed women had no efficacy or resulted in more disease. Although the authors did not provide results from this subset, they did report exploratory *post hoc* results stratified by age. In women aged 21–25 years in the total vaccinated cohort, vaccine efficacy was not significant against CIN3+ irrespective of associated HPV type, and there was an increase in HPV16- and HPV18-associated CIN3+ in the vaccinated group compared with the placebo group, although this difference was not significant.

"These results raise questions about the effectiveness and safety of vaccination of HPV-infected women."

Similarly, pooled results from North American trials using a vaccine against HPV16 or a quadrivalent vaccine (Gardasil) showed that vaccine efficacy was not significant in women with abnormal cytology results at baseline,

than would have occurred with protection against HPV16 and HPV18 alone[2]. The higher immunogenicity of Cervarix, compared with Gardasil, probably contributes to its greater cross-protection[10]. For both vaccines, the observed cross-protection rates may not apply to all populations, as overall protection will be lower (or higher) for a population that has a higher (or lower) rate of precancer caused by HPV types not protected by the vaccine. Since the neutralizing antibody titers against the protected nonvaccine HPV types are lower than those against HPV16 and HPV18 (ref. 10), cross-protection could wane faster than protection against HPV16 and HPV18 (ref. 11). In addition, the nonvaccine HPV types not protected by the vaccines tend to induce CIN3+ more slowly than the protected types[12], which implies that CIN3+ caused by slow progressor HPV types may account for a higher percentage of CIN3+ over time. These caveats about long-term cross-protection should not, however, obscure the ability of the HPV vaccines to reduce the majority of cervical and noncervical HPV-associated diseases, given the predominance of the vaccine HPV types in causing these diseases.

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and there was a nonsignificant increase in CIN2+ associated with HPV16 and HPV18 in the vaccinated group compared to the placebo group[13]. Together, these results raise questions about the effectiveness and safety of vaccination of HPV-infected women. Current guidelines from the US Centers for Disease Control and Prevention recommend catch-up HPV vaccination for women up to the age of 26 years regardless of the presence of HPV DNA, genital warts or abnormal cytology results[14]. Although subset analyses within trials must be interpreted with caution, these results suggest that outcomes from multiple trials stratified by clinically accessible baseline variables such as HPV16 and HPV18 DNA, number of sexual partners and cytology data would be useful in assessing the value of catch-up vaccination in older women.

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Cornelis J M Melief

These two studies[1,2] once again show that preventive vaccination with Cervarix provides close to 100% protection against disease induction by HPV16 and HPV18 represented in the vaccine but shows no efficacy in women with established persisting high risk infections with any type of HPV. Therefore, this vaccine is most effective in HPV-naive individuals. The vaccine also protected against premalignant disease and carcinoma induction by HPV31, HPV33, HPV45 and HPV51, which are related to HPV16 and HPV18 (ref. 2). This considerably widens the proportion of young women that could benefit from this preventive vaccine, although the protection against high-risk types not represented in the vaccine is incomplete, probably because the neutralizing antibodies elicited by vaccination are only partially cross-reactive against these other HPV types. These results call for intensified attempts to vaccinate all young women before sexual intercourse. Nevertheless, older women clearly benefit from this prevention, particularly if negative for high-risk HPV types at vaccination.

"It seems wise to await comparisons of the performance of such multivalent vaccines with Cervarix and Gardasil."

However, many issues remain to be addressed. How long does cross-reactive protection last against high-risk types other than HPV16 and HPV18? Conceivably, the antibody titers and affinity against these other HPV types are lower, causing earlier waning of cross-protection. Therefore, specific antibody responses should be followed over time to correlate specific antibody titers and affinity with level of protection. Apparently, the other HPV vaccine, Gardasil, shows similar cross-protection to Cervarix[3].

Currently, it seems unwise to omit cervical screening, because the duration of cross-protection and the performance of Ceravix and Gardasil are not known and because cross-protection with both of these vaccines is incomplete. In addition, experience worldwide has shown that compliance with screening and preventive vaccination programs is incomplete in most countries, even in highly developed ones, for reasons including religious, moral, social and financial ones. As multivalent vaccines based on L1 capsid recombinant proteins of multiple high-risk HPV types are also being developed, it seems wise to await comparisons of the performance of such vaccines with Cervarix and Gardasil. Nonetheless, these studies show that Cervarix is more efficacious than previously thought and is also safe in these large cohort studies[1,2].

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